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In re Application of:
Lauren Johnson, et al.

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For: ALFALFA PLANTS WITH DETECTABLE TANNIN LEVELS AND METHODS FOR PRODUCING SAME

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The undersigned hereby submits the following publications under 37 C.F.R. §§ 1.99 for consideration in connection with the above-referenced U.S. patent application:

Golpen, B.P., *et al.*, "A Search for Condensed Tannins in Annual and Perennial Species of *Medicago, Trigonella,* and *Onobrychis,*" *Crop Science*, Vol. 20, published November-December 1980

Peters, Darren J., *et al.*, "Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase

from trembling aspen (*Populus tremuloides*)," *The Plant Journal*, 32:701-712, published 2002

Treutter, D., "Chemical Reaction Detection of Catechins and Proanthocyanidins With 4-Dimethylaminocinnamaldehyde," *Journal of Chromatography*, 467:185-193, published 1989

Sarkar, Subodh K., *et al.*, "Specificity of the Vanillin Test for Flavanols," *J. Agric. Food Chem.*, Vol. 24, No. 2, published 1976

Schofield, P., *et al.*, "Analysis of condensed tannins: a review," *Animal Feed Science and Technology*, 91:21-40, published 2001

Skadhauge, Birgitte, *et al.*, "Leucocyanidin Reductase Activity and Accumulation of Proanthocyanidins in Developing Legume Tissues," *American Journal of Botany*, 84(4):494-503, published 1997

Lees, Garry L., "Condensed Tannins in Some Forage Legumes: Their Role in the Prevention of Ruminant Pasture Bloat," *Plant Polyphenols*, published 1992

Lees, Garry L., *et al.*, "Effect of High Temperature on Condensed Tannin Accumulation in Leaf Tissues of Big Trefoid (*Lotus uliginosus* Schkuhr)," *J. Sci. Fd Agric.*, 63:415-421, published 1994

Milić, Božidar Lj., "Lucerne Tannins," *J. Sci. Fd Agric.*, 23:1151-1156, published 1972

A copy of each of the above documents is enclosed herewith.

Pursuant to 37 C.F.R. §§ 1.17(p), a check in the amount of $180.00 is enclosed. Should any additional fee under 37 C.F.R. §§ 1.16 to 1.21 be deemed necessary for any reason relating to this document, the Commissioner is hereby authorized to deduct said fee from Fulbright & Jaworski, L.L.P. Deposit Account No. 50-1212/10203766.

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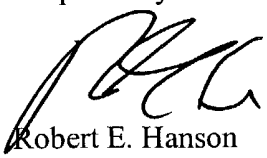

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A Search for Condensed Tannins in Annual and Perennial Species of *Medicago*, *Trigonella*, and *Onobrychis*[1]

B. P. Goplen, R. E. Howarth, S. K. Sarkar, and K. Lesins[2]

ABSTRACT

Bloat of ruminant animals feeding on fresh, green alfalfa (*Medicago sativa* L.) is caused by high levels of soluble plant proteins which act as foaming agents. It is postulated that bloat-safe cultivars may be bred by introducing condensed tannins (flavolans) which would act as protein precipitants to preclude foam formation and consequent bloat. Using the vanillin-HCl spot test, large populations of alfalfa were screened for tannins. These included several accessions, cultivars, and breeding populations. Extensive screening of 2n *M. falcata* L. and 4n *M. sativa* treated with the chemical mutagens ethyl methanesulfonate or ethyleneimine failed to reveal any mutations for tannins. Screening of 33 species (a total of 86 accessions) of annual *Medicago* was negative. Similarly, screening of 28 species (a total of 92 accessions) of perennial *Medicago* was also negative. Examination of 30 species (46 accessions) of the closely related *Trigonella* genus did not reveal any tannin-containing plants. On the other hand, testing of 123 accessions of 10 species of Sainfoin (*Onobrychis*) showed that all plants examined within this bloat-safe genus contained high levels of tannins.

Several of the common pasture legumes, including both bloat-causing and bloat-safe types, were examined for tannins in leaf tissue, flower (petal) tissue and seeds. All of these legumes except alfalfa and cicer milkvetch (*Astragalus cicer* L.) contained tannins in petal tissue. All of the legumes, including alfalfa, contained tannins in seedcoat tissue except that of a homozygous recessive, white-seeded strain of alfalfa.

The implications of breeding for tannins to provide a bloat-safe alfalfa are discussed.

Additional index words: **Flavolans, Pasture bloat, Protein precipitants, Alfalfa.**

PASTURE bloat in cattle is caused by the formation of a persistent foam that traps the gaseous products of fermentation in the reticulo-rumen (Howarth et al., 1977; Reid, 1960). Soluble plant proteins have been implicated as the main foaming agents responsible for the persistent bloating foams (Cooper et al., 1966; Jones et al., 1970; Mangan, 1959). Kendall (1966) first suggested that tannins in nonbloating legume forages may be responsible for preventing pasture bloat. He postulated that the nonbloating legumes contained tannins that precipitated the foam-producing soluble plant proteins and thus inhibited foam production. Cooper et al. (1966) made a survey of 27 legume species and found that nonbloating forages produced much smaller volumes of foam in vitro than forage samples from known bloating forages. Jones and Lyttleton (1971) later demonstrated a negative association between bloat and the presence of protein precipitants in leaves of 11 species of temperate legumes. Jones et al. (1973) subsequently showed that the protein precipitants were flavanol polymers (flavolans or condensed tannins, Swain and Bate-Smith, 1962). Based on this work (Jones et al., 1973), it was suggested that if a tannin-containing white clover (*Trifolium repens* L.) can be bred, pastures containing this legume would be nonbloating.

A general property of tannins is the ability to precipitate proteins. However, "tannins" is a general term often applied to low polymer phenols which may lack or possess only a limited capacity to precipitate proteins. In this paper, tannins is used according to the definition of Swain and Bate-Smith (1962) and refers to condensed tannins as found in herbaceous plants which have the property of precipitating proteins.

Experimental evidence indicates that condensed tannins prevent bloat by acting as protein precipitants. It would then appear possible to breed a bloat-safe alfalfa if tannins could be bred into this popular forage legume. Hence, an extensive screening program was launched to search for tannin-containing plants within common alfalfa (*Medicago sativa* L.). The screening program was later extended to include most of the annual and perennial species of *Medicago* and several closely related *Trigonella* spp. Several Sainfoins (*Onobrychis* spp.) were also screened to determine the relative frequency of tannin-containing plants in this high tannin, bloat-safe genus.

MATERIALS AND METHODS

Plant Materials

Medicago (alfalfa). Over 10,000 alfalfa plants in the breeding nurseries at Saskatoon were tested for tannin content using the vanillin-HCl spot test (Sarkar and Howarth, 1976). These plants included numerous acessions, cultivars, and breeding populations grown in the field as spaced plants, and occasionally as solid-seeded rows.

Following treatment with the chemical mutagens ethyleneimine or ethylmethanesulfonate, an M_2 population of *Medicago varia* Martin cv., 'Beaver,' consisting of approximately 20,000 plants, was screened for tannins. These plants came from an original source of 67 Beaver plants treated with chemical mutagens in an attempt to induce the desired mutation for tannin production. In a second population of a diploid *M. falcata* L. accession, approximately 6,000 plants were also screened for tannins. These plants originated from 250 plants that were treated with the above chemical mutagens. The resultant polycross seed was harvested and grown through two consecutive generations. Tannin screening was carried out on the second-generation polycross progenies. Selfing of the *M. falcata* plants was precluded because of low self-fertility. All the *M. falcata* plants were spaced 1 × 1 m in the field and tested in the 2nd year of growth. Similarly, approximately 6,000 plants of Beaver were screened in the field in their 2nd year of growth. An additional 14,000 seedlings 4 to 6 weeks old were screened in the greenhouse.

Thirty-three species comprising 86 accessions of annual *Medicago* were grown and tested for tannin content. Most of these

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species were evaluated at Saskatoon and Edmonton. At Edmonton, plants were grown under greenhouse conditions with a 16-hour photoperiod. Artificial light was provided by fluorescent and incandescent light sources, giving a PPFD of approximately 150 $\mu E \cdot sec^{-1}m^{-2}$. Greenhouse conditions were similar at Saskatoon except the photoperiod provided was 18 hours and the light source was Gro-lux fluorescent tubes and incandescent bulbs. The number of plants grown and tested for each species ranged from 5 to 20.

Species nomenclature and identity followed the scheme of Lesins and Gillies (1972). Other legumes species known to contain condensed tannins were grown and screened for tannins at the same time the annual *Medicago* spp. were screened at Saskatoon. These are listed in Table 1.

A total of 28 perennial *Medicago* spp., including 92 accessions were tested for tannin content. As with the annual species, most of these species were grown and tested independently under greenhouse conditions at Saskatoon and Edmonton. Greenhouse conditions were the same as those for the annual species. The number of plants grown and tested for each species varied widely, with a range of 5 to 30. Leaf tissue, petal tissue, and seeds of the following greenhouse-grown legumes were tested for condensed tannins: alfalfa (*M. varia*), red clover (*Trifollum pratense* L.), white clover (*T. repens* L.), sainfoin (*Onobrychis viciifolia* Scop.), birdsfoot trefoil (*Lotus corniculatus* L.), and cicer milkvetch (*Astragalus cicer* L.).

Trigonella. Thirty species of *Trigonella* were grown and tested for tannin content. Greenhouse conditions were similar to those for the *Medicago* spp. as above. A range of 5 to 12 plants were tested from each accession. This genus was included because of its close taxonomic relationship with *Medicago*, often resulting in intergeneric synonyms (Lesins and Gillies, 1972). All of the *Trigonella* accessions were supplied by the late A. T. H. Gross, Research Station, Brandon, Manitoba, and by Wilbur Robertson, Research Station, Ottawa, Ontario.

Onobrychis (sainfoin). Ten species of *Onobrychis* comprising 123 accessions, were grown in the field at Saskatoon. Each accession was seeded in single rows 3.1 m long and 1 m apart. In the 2nd year of growth, random leaf samples from five plants/row were tested for tannins. These accessions were kindly supplied by the Plant and Soil Science Dep., Montana State Univ., Bozeman.

Chemical Screening for Condensed Tannins

The vanillin-HCl screening test was used to screen plants for the presence of condensed tannins. For this test, new fully developed leaves near the upper part of the plant were crushed between two layers of Whatman No. 3 chromatography paper, and vanillin solution was applied to the imprint of plant sap on one layer of the paper (Jones et al., 1975). The vanillin solution contained two volumes of 10% w/v vanillin in ethanol mixed with one volume of concentrated HCl. A control solution (two volumes of ethanol in one volume of concentrated HCl) was applied to the imprint of the second layer to avoid the possibility of a false positive reaction (Sarkar and Howarth, 1976). Red color resulting from the presence of flavolans (condensed tannins) appears on the imprint treated with the complete reagent solution (vanillin plus HCl), but not on the imprint treated with the control solution (HCl).

RESULTS AND DISCUSSION

Medicago. No tannins were found in any of the 1,000's of accessions, varieties, or breeding populations of alfalfa. Similarly, no tannin-containing plants were found in 20,000 plants of 4n *M. sativa* alfalfa, nor 6,000 plants of 2n *M. falcata* alfalfa following treatment with chemical mutagens. The diploid (2n) *M. falcata* alfalfas were included in the mutation program because of the greater probability of expression of a tannin-containing phenotype in diploids in comparison to autotetraploid (4n) alfalfas if tannin production was based on a homozygous recessive genotype. However, tannin production is probably determined by genes with dominance, because interspecific studies in *Lotus* (Harney and Grant, 1964) indicated tannin inheritance by dominant gene(s). Similarly, Feenstra (1960) found that leucoanthocyanidins (procyanidins) were formed in the seedcoat of *Phaseolus vulgaris* only in the presence of the dominant gene *Sh*. Recessive white seeded alfalfa also lacks tannins in the seedcoat. In addition, the presence or absence of many metabolic products in plants is determined by a dominant:recessive relationship. If tannin production in alfalfa is determined by a dominant gene(s), detection of, and breeding for, tannins should be easily accomplished.

We were not successful in finding tannin-containing plants in either annual (Table 1) or perennial (Table 2) *Medicago* spp. The iclusion of several known legume species known to contain tannins revealed that the vanillin-HCl test was giving valid results and corroborated a previous study (Sarkar et al., 1976). These results are in agreement with those of Rumbaugh (1979) who made an extensive search of common alfalfas and 21 annual *Medicago* species, and failed to find any tannin-containing plants.

In striking contrast to the lack of tannins in the leaves of *Medicago* plants, all of the seeds of these and of the various other legume species examined had high levels of condensed tannins in the seedcoat (Table 2). This included seeds from the tannin-free, bloat-causing legumes (alfalfa, red clover, and white clover) as well as the tannin-containing, bloat-safe legumes (sainfoin and birdsfoot trefoil). Similarly, Jones et al. (1975) reported the presence of medium concentrations of tannins in petals of red clover and white clover as well as traces of tannins in the petioles of some of these plants. Electron microscopy showed the flavolans to be present in the vacuoles of epidermal cells. The presence of flavolans was further confirmed by [illegible]

Table 1. List of 86 accessions of 33 annual *Medicago* spp. that gave negative reactions for flavolans (condensed tannins) in leaves using the vanillin-HCl spot test, compared to species that gave positive reactions.

Annual *Medicago* spp.	No. accessions tested	Annual *Medicago* spp.	No. accessions tested
M. aculeata Willd.	1	*M. polymorpha* L.	5
M. arabica Huds.	5	*M. praecox* DC	1
M. blancheana Boiss.	1	*M. radiata* L. (synonym *Trigonella radiata* Boiss.)	3
M. ciliaris All.	3	*M. rigidula* Desr.	1
M. constricta Dur.	1	*M. rotata* Boiss.	5
M. coronata Bart.	5	*M. rugosa* Desr.	1
M. disciformis DC	1	*M. secundiflora* Durieu	1
M. granadensis Willd.	1	*M. shepardii* Post.	1
M. intertexta Mill.	1	*M. sauvagei* Negre	1
M. laciniata Mill.	1	*M. scutellatta* Mill.	1
M. laniigera Winkl. & Fedtsch.	1	*M. soleirolii* Duby	9
M. littoralis Rhode	9	*M. tenoreana* Ser.	8
M. lupulina L.	8	*M. tornata* Mill.	6
M. minima Bart.	6	*M. truncatula* Gaertn.	1
M. murex Willd.	1	*M. turbinata* All.	1
M. muricoleptis Tin.	1		
M. noeana Boiss.	2		
M. orbicularis Bart.	8		

Check species known to contain condensed tannins:
Sainfoin, *Onobrychis viciifolia* Scop.
Crownvetch, *Coronilla varia* L.
Lespedeza, *Lespedeza cuneata* Don.
Birdsfoot trefoil, *Lotus corniculatus* L.
Rabbit foot clover, *Trifolium arvense* L.
Large hop clover, *Trifolium campestre* Schreb.
Small hop clover, *Trifolium dubium* Sibth.

Table 2. List of 92 accessions of 28 perennial *Medicago* spp. that gave negative reactions for flavolans (condensed tannins) in leaves using the vanillin-HCl spot test.

Perennial *Medicago* spp.	No. accessions tested	Perennial *Medicago* spp.	No. accessions tested
M. arborea L.	3	*M. papillosa* Boiss.	2
M. cancellata M.B.	2	*M. pironae* Vis.	3
M. carstiensis Wulf.	2	*M. platycarpa* Trautv. (synonym *Trigonella platycarpos* L.)	2
M. cretacea Grosch. (synonym *Trigonella cretacea* Taliev.)	2	*M. prostrata* Jacq.	2
M. daghestanica Rupr.	2	*M. rhodopea* Velen.	2
M. drawakhetica Bordz.	2	*M. rupestris* M.B.	2
M. falcata L.	10	*M. ruthenica* Ledeb. (synonym *Trigonella ruthenica* L.)	1
M. glomerata Balb.	3	*M. sativa* L.	20
M. glutinosa M.B.	3	*M. sativa*, subsp. *sativa* L.	6
M. hemicycla Grossh.	1	*M. sativa*, subsp. *coerulea* Schmalh.	1
M. hybrida Trautv. (synonym *Trigonella hybrida* Pourr.)	2	*M. saxatilis* M.B.	2
M. lupulina L.	4	*M. schlechtalii* Sumn.	1
M. marina L.	3	*M. suffructicosa* subsp. *suffructicosa* Urb.	1
		M. suffructicosa subsp. *leiocarpa* Urb.	5
		M. varia Martin	10

Table 3. Occurrence (+) or non-occurrence (−) of tannins in leaf tissue, petal tissue and seeds of several common bloating and nonbloating pasture legumes.

Legume tested	Farm classification	Vanillin-HCl test reaction: Leaf tissue	Flower (petal) tissue	Seeds
Medicago sativa L.	bloating	−	−	+ (normal seeds) − (white seeds)†
Trifolium pratense L.	bloating	−	+	+
T. repens L.	bloating	−	+	+
Onobrychis viciifolia Scop.	bloat-safe	+	+	+
Astragalus cicer L.	bloat-safe	−	−	+
Lotus corniculatus L.	bloat-safe	+	+	+

† From an 8-clone white-flowered homozygous recessive (*w w w w*) synthetic developed by Goplen for isolation distance studies.

in solubility tests with gel electrophoresis. We confirmed the presence of tannins in white and red clover petals, but were unable to find any tannins in alfalfa flowers taken at random from greenhouse-grown plants. However, all alfalfa seeds had tannins in their seedcoat (except the white seedcoat genotype).

Trigonella. None of the 30 species of *Trigonella* examined contained condensed tannins (Table 4). This tends to corroborate the close phylogenetic relationship of *Trigonella* and *Medicago* (Lesins and Lesins, 1972), because none of the *Medicago* species contained tannins.

Onobrychis. All of the *Onobrychis* species and accessions examined contained high levels of tannins (Table 5). The vanillin-HCl test was semi-quantitative; the strong color reaction on all plants tested indicated high levels of tannin.

GENERAL DISCUSSION

Pasture bloat is caused by high levels of soluble plant proteins which act as foaming agents. There is general agreement that tannins in forages prevent bloat through their action as protein precipitants, thus essentially preventing the soluble proteins from acting as foaming agents. Although tannins have not been found in leaf tissues of common alfalfa or exotic *Medicago* species, it was of considerable significance to find condensed tannins in the seedcoat of all the alfalfa seeds examined (except the white seedcoat genotype). Seedcoat tissue is of the same embryological origin and is genetically identical to the leaf tissue which lacks tannins in alfalfa. Thus, the genetic mechanism for tannin production appears to already exist in alfalfa. It then remains for the geneticist to search for spontaneous or induced mutations to cause expression of the tannin gene(s) at an earlier ontological stage so that tannins would also be present in leaf tissue. The routine screening of large alfalfa populations in this study and that of Rumbaugh (1979) indicate the futility of this approach. Interspecific hybridization is also precluded because no species of *Medicago* or of the closely related *Trigonella* contain tannins. The mutation approach may be the most feasible because the modified vanillin-HCl test is

Table 4. List of 46 accessions of 30 *Trigonella* spp.† that gave negative reactions for flavolans (condensed tannins) in leaves using vanillin-HCl spot test.

Trigonella spp.	No. accessions tested	*Trigonella* spp.	No. accessions tested
1. *T. anguina* Delile	1	18. *T. meliotus-corniculata* (L.) Hylander (synonym *T. corniculata*)	1
2. *T. arabica* Delile	2	19. *T. monspeliaca* L.	1
3. *T. arcuata* C. A. Meyer	1	20. *T. monantha* C. A. Meyer	1
4. *T. brachycarpa* (Fisch.) Moris	1	21. *T. noeana* Boiss.	1
5. *T. calliceras* Fisch.	5	22. *T. ornithopodioides* DC.	1
6. *T. coelesyriaca* Boiss.	1	23. *T. platycarpos* L.	1
7. *T. coerulea* (L.) Ser.	8	24. *T. polycerata* L.	2
8. *T. corniculata* (L.) L.	2	25. *T. procumbens* (Besser) Reichb.	1
9. *T. cretica* (L.) Boiss.	8	26. *T. radiata* (L.) Boiss. (synonym *Medicago radiata* L.)	3
10. *T. emodi* Benth.	1	27. *T. rigida* Boiss.	1
11. *T. foenum-graecum* L.	4	28. *T. spicata* Sibth. & Sm.	1
12. *T. gladiata* Stev.	1	29. *T. suavissima* Lindl.	1
13. *T. hamosa* L.	1	30. *T. uncata* Boiss. et Noe	1
14. *T. incisa* Benth.	1		
15. *T. kotschyi* Fenzl	1		
16. *T. lipskyi* Sir.	1		
17. *T. meliloto-coerulaus* (L.) Aschers. & Graebner (synonym *T. coerulea*)	2		

† Authority citations follow Sirjaev (1933) except for nos. 7, 26, 27 which follow Vasil'Chenko (1953), and for nos. 4, 9, 25 which follow Huber-Morath (1970).

Table 5. List of 123 accessions of 10 *Onobrychis* spp.† that gave positive reactions for flavolans (condensed tannins) in leaves using the vanillin-HCl spot test.

Onobrychis spp.	No. accessions tested
1. *O. argentea* Boiss.	1
2. *O. arenaria* (Kit. ex Willd.) DC	12
3. *O. hajastana* Grossheim	1
4. *O. inermis* Stev.	3
5. *O. megataphros* Boiss.	1
6. *O. tanaitica* Spr. (synonym *O. arenaria*)	5
7. *O. transcaucasica* Grossh.	50
8. *O. vaginalis* C. A. Meyer	1
9. *O. viciifolia* Scop.	47
10. *O.* spp.	2

† Authority citations are as follows: 1, 2 from Index Kewensis, 3, 4, 6, 8, 10 from C. Hedge (1970 *In* op. cit. for *Trigonella*, p. 560–589), 5, 6, 9 from Sirjaev 1925 to 1926, and 7 from Flora Europaea Vol. 2.

rapid and permits the screening of large populations with minimum time and effort. Protoplast fusion may eventually provide the means for an intergeneric transfer of genes for desirable tannins from sainfoin or crownvetch (*Coronilla varia* L.) to alfalfa. The universal occurrence of tannins in all of the *Onobrychis* species examined in this study is encouraging to this approach.

Tannins in plants act as effective repellents to animal and microbial predators or parasites. An important property is their ability to precipitate proteins which render the tannin-containing tissues unpalatable by precipitating salivary proteins, or by inactivating enzymes, thus impeding the invasion of the tissues of the host by the parasite (Bate-Smith, 1973). Tannins would therefore be of selective value in an evolutionary sense in protecting the plant from predators (e.g. birds, small animals), and disease organisms (Harris and Burns, 1973). Possibly this accounts for the common occurrence of tannins in the seedcoats of various legume seeds.

ACKNOWLEDGMENT

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REFERENCES

Bate-Smith, E. C. 1973. Haemanalysis of tannins: the concept of relative astringency. Phytochemistry 12:907-912.

Cooper, C. S., R. F. Eslick, and P. W. McDonald. 1966. Foam formation from extracts of 27 legume species in vitro. Crop Sci. 6:215-216.

Feenstra, W. J. 1960. Biochemical aspects of seedcoat colour inheritance in *Phaseolus vulgaris* L. Meded. Landbouwhogesch., Wageningen 60:1-53.

Harney, P. M., and W. F. Grant. 1964. A chromatographic study of the phenolics of species of *Lotus* closely related to *L. corniculatus* and their taxonomic significance. Am. J. Bot. 51:621-627.

Harris, H. B., and R. E. Burns. 1973. Relationship between tannin content of sorghum grain and preharvest seed molding. Agron. J. 65:957-959.

Howarth, R. E., W. Majak, D. E. Waldern, S. A. Brandt, A. T. Fesser, B. P. Goplen, and D. T. Spurr. 1977. Relationship between ruminant bloat and the chemical composition of alfalfa herbage: I. Nitrogen and protein fractions. Can. J. Anim. Sci. 57:345-357.

Huber-Morath, A. 1970. *Trigonella* L. *In* P. H. Davis (ed.) Flora of Turkey and the East Aegean Islands. 3:452-467. University Press, Edinburgh.

Jones, W. T., L. B. Anderson, and M. D. Ross. 1973. Bloat in cattle. XXXIX. Detection of protein precipitants (tannins) in legumes. N. Z. J. Agric. Res. 16:441-446.

———, and J. W. Lyttleton. 1971. Bloat in cattle. XXXII. A survey of legume forages that do and do not produce bloat. N. Z. J. Agric. Res. 14:101-107.

———, ———, and R. T. J. Clarke. 1970. Bloat in cattle. XXXIII. The soluble proteins of legume forages in New Zealand and their relationship to bloat. N. Z. J. Agric. Res. 13:149-156.

Kendall, W. A. 1966. Factors affecting foams with forage legumes. Crop Sci. 6:487-489.

Lesins, K., and C. B. Gillies. 1972. Taxonomy and cytogenetics of *Medicago*. p. 53-86. *In* C. H. Hanson (ed.) Alfalfa Science and Technology. Am. Soc. of Agron., Madison, Wis.

Mangan, J. L. 1959. Bloat in cattle. XI. The foaming properties of proteins, saponins and rumen liquor. N. Z. J. Agric. Res. 2:47-61.

Reid, C. S. W. 1960. Bloat: The foam hypothesis. Proc. 8th Int. Grassland Congr., p. 668-672.

Rumbaugh, M. D. 1979. The search for condensed tannins in the genus *Medicago*. Am. Soc. Agron. Abstr., p. 75.

Sarkar, S. K., and R. E. Howarth. 1976. Specificity of the vanillin test for flavanols. J. Agric. Food Chem. 24:317-320.

———, ———, and B. P. Goplen. 1976. Condensed tannins in herbaceous legumes. Crop Sci. 16:543-546.

Sirjaev, G. 1933. Generis *Trigonella* L. revisio critica. (In Latin). VI. Fac. Sci. Univ. Masaryk, Brno, Czechoslovakia. 87 p.

Swain, T., and E. C. Bate-Smith. 1962. Flavonoid compounds. p. 755-809. *In* A. M. Florkin and H. S. Mason (ed.) Comp. Biochem. 3. Academic Press Inc., N.Y.

Vasil'Chenko, I. T. 1953. Obzor vidov roda *Trigonella* L. Trudy Botanischeskogo Instituta im. V. L. Komarova Akademii Nauk SSSR, Seriya 1, vyp. 10:124-269.

Evaluation of Foreign and Domestic Cotton Cultivars and Strains for Boll Weevil Resistance[1]

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ABSTRACT

Forty-four domestic and introduced cotton (*Gossypium hirsutum* L.) cultivars and strains were evaluated in the laboratory for resistance to the boll weevil, *Anthonomus grandis* Boh. Oviposition by the boll weevil was significantly lower in squares (flower buds) from eight cottons ('Lasani 11,' 'AC 134,' 'Albar 627,' G077-2, 'BP 52/NC 63,' TX-LY-18-72, DES-HERB 16, and DES-ARB 16) than in squares from the commercial cultivar, 'Deltapine 16.' Five of the eight were introductions. Oviposition was not significantly lower in any entry than in 'Stoneville 213,' another commercial cultivar. There was no significant correlation between terpenoid aldehyde content in squares of 10 entries and rate of oviposition. The potential of the entries in breeding for increased resistance to boll weevil is discussed.

Additional index words: Anthonomus grandis Boh., *Gossypium hirsutum* L., Oviposition suppression, Terpene content.

THE larva and adult boll weevil (*Anthonomus grandis* Boh.) feed on the cotton square (flower bud). Adults chew through the calyx and unopened petals of the square and feed on anthers. Eggs are deposited through these feeding holes. Abscission of the square 5 to 9 days after oviposition results from larval feeding and developing within the square (Hunter and Pierce, 1912; Coakley et al., 1969). Abscission also results in

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Molecular analysis of herbivore-induced condensed tannin synthesis: cloning and expression of dihydroflavonol reductase from trembling aspen (*Populus tremuloides*)

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Summary

In order to study condensed tannin synthesis and its induction by herbivory, a dihydroflavonol reductase (DFR) cDNA was isolated from trembling aspen *(Populus tremuloides)*. Bacterial overexpression demonstrated that this cDNA encodes a functional DFR enzyme, and Southern analysis revealed that *DFR* likely is a single-copy gene in the aspen genome. Aspen plants that were mechanically wounded showed a dramatic increase in *DFR* expression after 24 h in both wounded leaves and unwounded leaves on wounded trees. Feeding by forest tent caterpillar (*Malacosoma disstria*) and satin moth (*Leucoma salicis*) larvae, and treatment with methyl jasmonate, all strongly induced *DFR* expression. DFR enzyme activity was also induced in wounded aspen leaves, and phytochemical assays revealed that condensed tannin concentrations significantly increased in wounded and systemic leaves. The expression of other genes involved in the phenylpropanoid pathway were also induced by wounding. Our findings suggest that the induction of condensed tannins, compounds known to be important for defense against herbivores, is mediated by increased expression of *DFR* and other phenylpropanoid genes.

Keywords: plant defense, herbivory, proanthocyanidins, forest tree.

Introduction

Plants have evolved both defense proteins and phytochemicals to defend themselves against herbivores, and plant defense is thought to be a driving force in the evolution of phytochemical diversity in the plant kingdom. Although many defenses are constitutively expressed, they may also be induced, and are thus produced only after wounding or herbivore damage (Karban and Baldwin, 1997). One of the earliest observations of herbivore-induced phytochemical defense was the increased accumulation of tannins in red oak leaves on trees that had been defoliated by gypsy moth (*Lymantria dispar*) (Schultz and Baldwin, 1982). Tannins are large polyphenolic compounds, typically found in woody plants, and have strong biological effects due to their protein-binding ability. Two biosynthetic types of tannin are known, the condensed tannins (CTs), also known as proanthocyanidins, and the hydrolyzable tannins (Haslam, 1993). Ingestion of tannins can have strong negative effects on herbivorous insects, for example a reduced efficiency in nutrient absorption and midgut lesions (Hagerman and Butler, 1991). The type and degree of antiherbivore activity depends on the structure (plant source) of the tannin, as well as the biochemical conditions within the herbivore gut, which can differ dramatically between insect species (Ayres *et al.*, 1997; Barbehenn and Martin, 1994). Concentrations of tannins vary widely among plants; furthermore, in species such as willow, birch, and aspen, an induction by herbivory or wounding has been observed, suggesting that tannin induction may be an important induced defense in some plants (reviewed in Constabel, 1999). However, very little is known about the mechanism of induced tannin accumulation, and this phenomenon has not yet been investigated at the level of gene expression.

In order to study the molecular biology of induced CT biosynthesis, we chose trembling aspen (*Populus tremuloides* Michx.) as an experimental system. Leaves of this species contain as much as 18% DW of CTs in leaves, and respond to damage with an induced accumulation of these chemicals (Lindroth and Hwang, 1996; Osier and Lindroth,

2001). Trembling aspen is a widespread North American forest tree, with significant economic and ecological importance in the boreal forest of Canada. It is susceptible to defoliation by leaf-eating insects such as the forest tent caterpillar (*Malacosoma disstria*), large aspen tortrix (*Choristoneura conflictana*), and gypsy moth (Ives and Wong, 1988). Preliminary data indicated a strong negative effect of aspen leaf CT concentration on the growth rates of forest tent caterpillar larvae (C.P. Constabel and J. Spence, unpublished data), confirming earlier reports of the potential importance of these secondary metabolites in aspen defense (Hwang and Lindroth, 1997). Differences in field susceptibility of two *Populus* clones to pest insects was also ascribed to differences in CT levels (Gruppe *et al.*, 1999). Other known biochemical defenses of aspen against herbivorous insects include several Kunitz trypsin inhibitor proteins and the antinutritive enzyme polyphenol oxidase (PPO), which are both strongly wound and herbivore induced (Haruta *et al.* 2001a,b). In addition to the high levels of CTs mentioned above, aspen leaves also contain the phenolic glycosides such as salicortin and tremulacin (Lindroth and Hwang, 1996). These are known to negatively impact larval performance of the forest tent caterpillar and gypsy moth (Hwang and Lindroth, 1997).

The biosynthetic pathway leading to CTs is well established, and many of the enzymes have been cloned; this provided an opportunity for investigating CT synthesis at the molecular level in a system with a well-characterized induced defense response. We focused on the enzyme dihydroflavonol reductase (DFR), the second last characterized

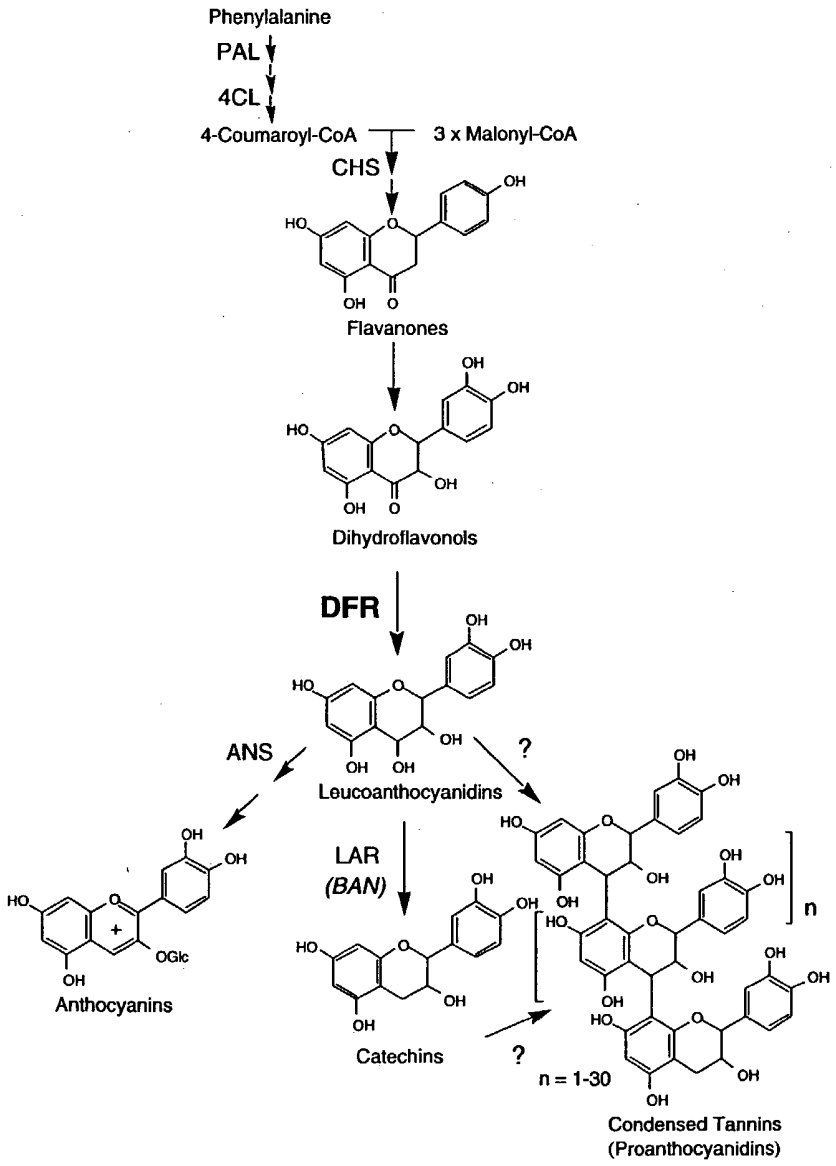

Figure 1. Outline of flavonoid biosynthetic pathway leading to the synthesis of condensed tannins (proanthocyanidins).
Enzyme names are abbreviated as follows: phenylalanine ammonia-lyase (PAL), 4-coumarate CoA ligase (4CL), chalcone synthase (CHS), dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), leucoanthocyanidin reductase (LAR), a likely homolog of the *Arabidopsis BANYULS* (*BAN*) gene. '?' represents the unidentified condensing enzyme that is proposed to polymerize leucoanthocyanidins and catechins to produce the condensed tannin polymer. In aspen, the polymer has an average length of seven monomers (Ayres *et al.*, 1997). Only the procyanidin-type monomer is shown.

enzymatic step in CT synthesis (Figure 1). Evidence that strongly links CT biosynthesis to DFR has been provided by mutant barley and *Arabidopsis* plants that are unable to accumulate anthocyanins and CTs due to inactivation of the *DFR* gene (Olsen *et al.*, 1993; Shirley *et al.*, 1995). The objectives of this study were to clone and characterize the expression of DFR, a key enzyme for CT synthesis, and to correlate *DFR* expression with CT accumulation and the expression of other defense and phenylpropanoid genes. We demonstrate that mechanical wounding, insect herbivory, and methyl jasmonate (MeJa) treatment all induced *DFR* expression in aspen leaves. Furthermore, we show that DFR activity and CT concentrations are inducible in wounded aspen foliage. These findings suggest that CT synthesis, mediated by increased expression of *DFR* and other phenylpropanoid enzymes, is an inducible defense in trembling aspen.

Results

Isolation and characterization of DFR from trembling aspen

DFR is a key enzyme involved in the synthesis of CTs, and the isolation of a full-length *DFR* cDNA would provide an important tool for analyzing induction of CT synthesis at the molecular level. A cDNA library constructed from wounded aspen leaves (Haruta *et al.*, 2001a) was screened with a PCR-generated *DFR* probe, and nine positive clones were excised and sequenced. Five identical clones had high sequence similarity to *DFR* genes from other plants. One cDNA clone, *DFR14a*, was completely sequenced on both strands, shown to encode a full-length DFR, and was re-named *PtDFR*. The nucleotide sequence of *PtDFR* is 1041-bp long and predicted to encode a protein of 346 amino acid residues with a molecular mass of 38.8 kDa (Figure 2).

Sequence comparison of *PtDFR* with sequence databases showed the highest identity with DFR proteins from *Vitis vinifera* (76.0%), *Malus* × *domestica* (74.0%), *Rosa hybrida* (72.0%), and *Fragaria* × *ananassa* (71.6%) (Figure 2). Analysis of *PtDFR* indicated that the highly conserved, putative N-terminal NADPH-binding domain found in other *DFR* genes (Lacombe *et al.*, 1997) was also present (Figure 2). Computational analysis using PSORT predicted that PtDFR is a membrane-associated enzyme, with no N-terminal signal sequence. Interestingly, amino acid-134 in the substrate specificity region was predicted to be an asp residue in PtDFR, rather than the asn found in most other plant DFRs. This residue is proposed to be important in substrate specificity, and may indicate that PtDFR preferentially

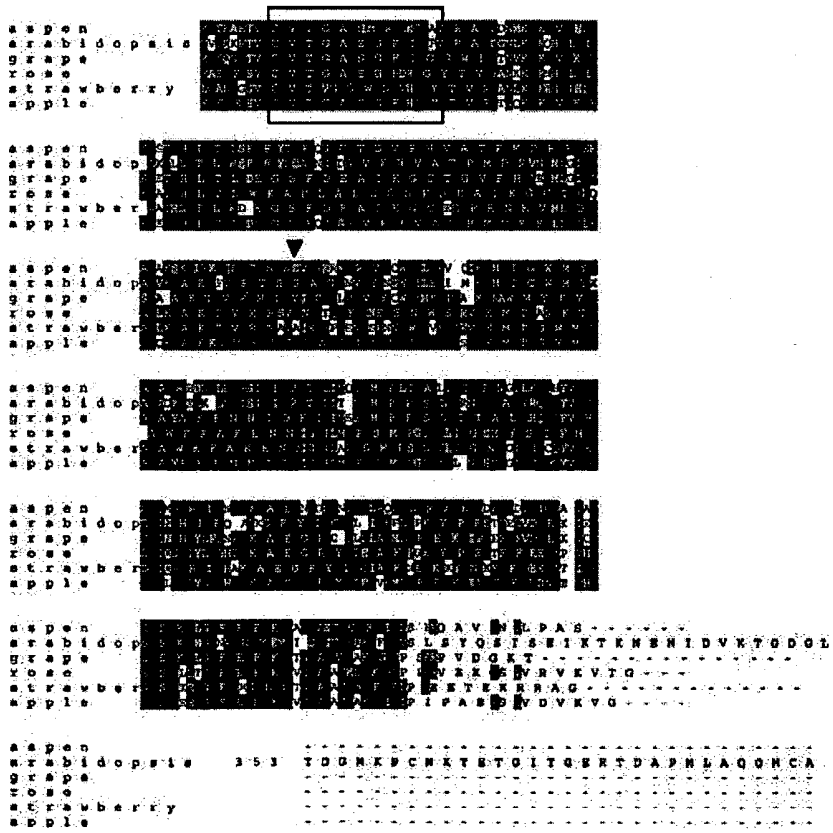

Figure 2. Multiple sequence alignment of the predicted PtDFR amino acid sequence with other DFR sequences.
Identical amino acids are shown in black (80%); similar amino acids are shown in grey. Accession numbers are AY147903 for trembling aspen (ptDFR), AF117268 for apple (*Malus* × *domestica*), Y11749 for grape (*Vitis vinifera*), D85102 for rose (*Rosa hybrida*), AF029685 for strawberry (*Fragaria* × *ananassa*), and AB007647 for *Arabidopsis thaliana*. Alignments were performed using the ClustalW 1.8 multialignment tool (http://searchlauncher.bcm.tmc.edu) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html). The triangle indicates the asp residue thought to be important for substrate specificity, and the highly conserved NADPH-binding region is boxed.

accepts dihydroquercetin and dihydromyricetin as substrates (Johnson *et al.*, 2001) (see Discussion).

In order to show that *PtDFR* encodes an enzyme with DFR activity, this cDNA was expressed in *Escherichia coli*. Cultures containing the *PtDFR* expression vector were induced with IPTG, sonicated, and the supernatant was used in DFR assays. All *E. coli* colonies tested that harbored the *PtDFR* cDNA displayed high DFR activity (0.07–0.08 nmol h^{-1} mg^{-1} protein). Control cultures containing only the pQE30 bacterial expression vector or an unrelated construct showed no DFR activity. Therefore, we conclude that *PtDFR* encodes a functional DFR enzyme.

The size of the *DFR* gene family in aspen was investigated using Southern analysis. Only a single band was detected on Southern blots washed at high stringency (Figure 3a), while low-stringency washes revealed the presence of three or four minor bands that hybridized with the *PtDFR* probe (Figure 3b). This suggests that aspen contains a single *DFR* gene, plus one or two *DFR*-like genes. The enzyme leucoanthocyanidin reductase, which is immediately downstream of DFR in CT synthesis, is encoded by a *DFR*-like gene (*BAN* in *Arabidopsis*) (Devic *et al.*, 1999), and therefore we speculate that these bands may represent *BAN* homologs of aspen (see Discussion).

Figure 3. Southern blot analysis of *DFR*.
Genomic DNA was restricted, electrophoresed, and hybridized with the *PtDFR* cDNA as described in the Experimental procedures.
(a,b) The same blot washed at high or low stringency, respectively. H = *Hin*dIII; N = *Nco*I; X = *Xba*I; E = *Eco*RI; U = uncut DNA.

Wound induction of DFR and CTs

Since aspen has a strong inducible defense response, we used *PtDFR* as a probe to determine if *DFR* expression is induced by simulated herbivory. Previously, we had shown that wounding of aspen leaves with a hemostat was effective in inducing the expression of known defense genes (Haruta *et al.*, 2001a,b), thus at least partially mimicking herbivore damage. Leaves of LPI 16–19 on young aspen plants were wounded, and leaves at LPI 12–15 were designated for systemic sampling. Leaves in this range are fully expanded, source leaves; they were chosen to avoid potential developmental effects seen in younger leaves which could mask responses to wounding. Preliminary experiments showed leaves from LPI 10–20 to have stable constitutive CT levels within the time frame of our experiments (data not shown).

Following wounding with a hemostat, both the wounded and unwounded (systemic) leaves were harvested at appropriate times, and RNA was extracted and analyzed by Northern hybridization. At the beginning of the time course, the leaves had low but consistently detectable levels of *DFR* mRNA; however, after mechanical wounding, *DFR* mRNA was very abundant in both wounded and systemic leaves (Figure 4a). Induced expression of aspen *DFR* was apparent as early as 12 h after wounding, peaked after 24 h in both the wounded and systemic leaves, and then declined (Figure 4a). For comparison with other defense genes, the blot was stripped and re-hybridized with aspen polyphenol oxidase (*PtPPO*), a known wound-inducible gene in aspen and poplar (Constabel *et al.*, 2000; Haruta *et al.*, 2001b). The kinetics of *PPO* mRNA induction were virtually identical to those for *DFR*, although *PPO* showed no constitutive expression. Thus, our Northern analysis suggested that mechanical leaf damage strongly induced aspen *DFR* mRNA expression, and that this induction was systemic and co-ordinate with other aspen defense genes.

If the wound-induced expression of *DFR* mRNA we observed is to be indicative of a role in herbivore defense, both DFR activity and the CT concentration are predicted to increase following leaf damage. We therefore performed DFR assays on time course experiments using de-salted enzyme extracts prepared from control, wounded, and systemically wounded leaves. DFR activity increased several-fold after wounding, peaking at 48 h and then declining rapidly (Figure 4b). The kinetics of induction are consistent with the mRNA induction, which occurs earlier and peaks at 24 h after wounding. This experiment showed that DFR enzyme activity, like *DFR* mRNA, is locally and systemically induced by wounding in aspen leaves.

Next, we tested if CTs, the ultimate products of DFR and the flavonoid pathway in aspen leaves, also increased in leaves following wounding. For this experiment, leaves of LPI 12–19 were wounded and sampled as for the previous Northern analysis (Figure 4a). Concentrations of CTs in leaf

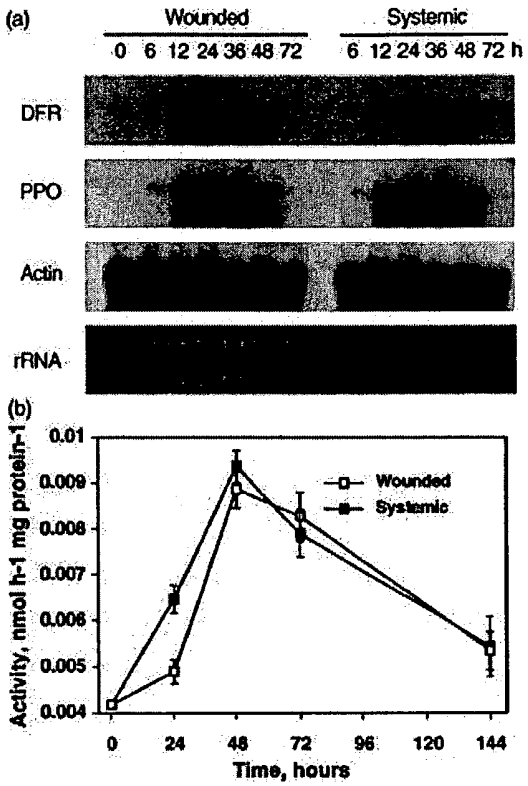

Figure 4. Analysis of *DFR* induction by simulated herbivory.
(a) Northern analysis. Both wounded leaves and unwounded leaves on wounded plants (systemic) were harvested at the appropriate times, total RNA was extracted, and analyzed on RNA blots. Following hybridization with a ^{32}P-labelled *PtDFR* probe, the blot was stripped and hybridized with poplar polyphenol oxidase (*PPO*) and actin. The *DFR* blot was exposed to X-ray film for 2 h, while both the *PPO* and actin blots were exposed for 24 h. The ethidium bromide-stained gel is shown as a loading control.
(b) Analysis of DFR activity in wounded and systemically wounded aspen leaves. Leaf proteins were extracted, de-salted, and assayed for DFR activity as described in Experimental procedures. Each point is the mean of three plants assayed; bars represent the standard error.

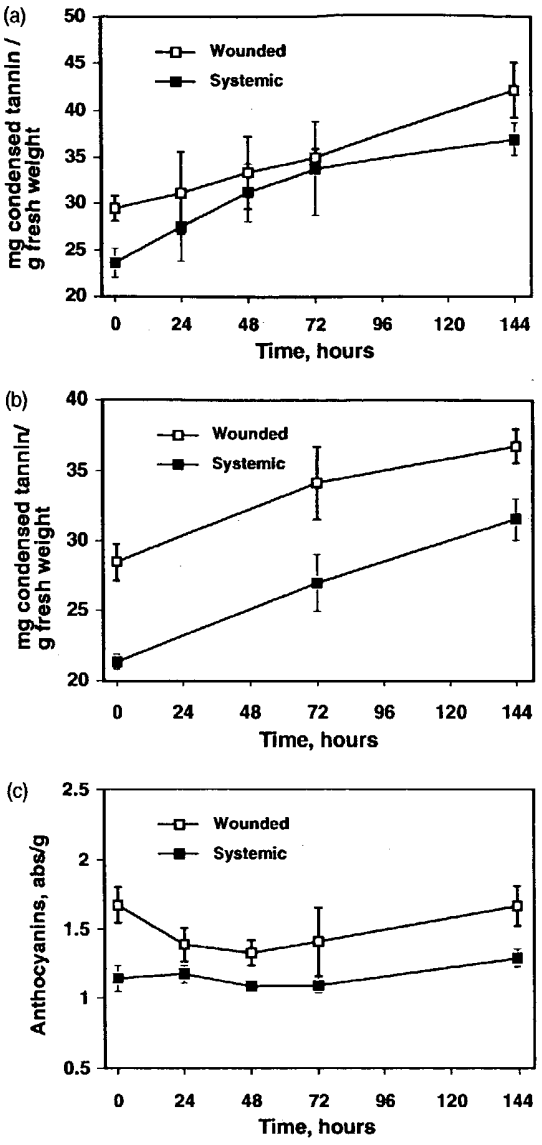

Figure 5. CT and anthocyanin concentrations in aspen leaves at different times after mechanical wounding.
Acetone and methanol extracts were prepared and assayed as described in the Experimental procedures. Each point is the mean of five plants assayed; bars represent the standard error.
(a) Butanol:HCl CT assays. For wounded leaves, the 144-h mean is significantly different from both 0- and 24-h means (LSD: $P=0.013$ and 0.021, respectively). For systemic leaves, only the 0-h control mean is significantly different from the 144-h means (LSD: $P=0.016$).
(b) Radial diffusion BSA precipitation assays. Both 72- and 144-h means are significantly different from the 0-h controls in wounded leaves (LSD: $P=0.036$ and 0.003, respectively) as well as in systemic leaves (LSD: $P=0.036$ and 0.001, respectively).
(c) Anthocyanin concentrations from the same leaf samples as in (a,b).

acetone extracts were determined using the *n*-butanol:HCl assay (Porter *et al.*, 1986) with purified sainfoin tannin as a CT standard (Koupai-Abyazani *et al.*, 1993). In both the wounded and systemic leaves, condensed tannin concentration increased after wounding (Figure 5a). Some of the values had large standard errors due to the variation in baseline CT concentrations in different individual trees. However, analysis of variance (two-way ANOVA) confirmed that the increases in CT concentrations over time were significant ($F_{4,38}=4.408$; $P=0.005$). While the treatment effects were not significant in this analysis ($F_{1,38}=3.026$; $P=0.090$), CT concentrations tended to be higher in wounded leaves harvested at 144 h, which is consistent with the wound-induced increases in *DFR* mRNA and DFR activity.

Tannins act as an antinutritive defense based on their ability to form complexes with proteins; these are often insoluble and can be detected as precipitated complexes. We therefore used the radial diffusion protein precipitation assay as a complementary method for estimating CT levels in aspen leaf extracts (Hagerman, 1987). This provided a

second assay method for CTs, and also emphasizes one of the biologically relevant properties of the induced tannins. The protein precipitation assays confirmed that tannin concentrations were higher in wounded and systemically wounded leaf extracts than in control extracts (Figure 5b); two-way ANOVA again indicated a significant change in protein-precipitating capacity over time ($F_{2,22} = 13.540$; $P < 0.001$), as well as a significant treatment effect ($F_{1,22} = 20.56$; $P < 0.001$), demonstrating wound-induced increases at the 72- and 144-h time points. Although the actual CT concentrations obtained with the radial diffusion method were slightly lower than those obtained using the *n*-butanol:HCl assay, overall the results were very similar. Therefore, we are confident that the increases in CTs we observed are relevant and likely to be detrimental to insects feeding on induced foliage.

As an additional control, we investigated if wounding could induce anthocyanin accumulation, since DFR is also important for anthocyanin synthesis. In the same time period when CT concentrations increased, anthocyanin concentrations in leaves did not increase significantly ($F_{4,38} = 1.644$; $P = 0.183$) (Figure 5c). In some experiments, concentrations of anthocyanins initially declined and then returned to constitutive levels or remained low; however, in none of our experiments, did we observe any anthocyanin induction. Thus, we conclude that the wound response in aspen leads to the products of DFR being channeled into tannin, but not anthocyanin synthesis.

Expression of phenylpropanoid genes

The wound-induced increase in CT accumulation predicts that the entire phenylpropanoid pathway should be upregulated by wounding. Therefore, we investigated the expression of several key phenylpropanoid genes in response to wounding. The expression of phenylalanine ammonia lyase (*PAL*), 4-coumarate-CoA ligase (*4CL*), and chalcone synthase (*CHS*) have been shown to be induced by wounding and stress treatments in other plant species (Dixon and Paiva, 1995; Hahlbrock and Scheel, 1989). Probes for *PAL*, *4CL*, and *CHS* were obtained by PCR amplifying and cloning cDNAs encoding these enzymes from an aspen cDNA library using sequence information in GenBank. These cloned PCR products were confirmed by sequencing and then used to generate labeled probes for hybridizing with the previous time course experiments. All three genes were wound induced, with kinetics similar to *DFR*, although the induction appeared less dramatic (Figure 6). Maximum expression was generally at 24 h after wounding, followed by a decline to constitutive levels after 72 h. In systemic tissue, mRNA levels of *PAL* and *4CL* also reached a maximum at 24 h, while expression levels of *CHS* were highest after 36 h. Interestingly, *PAL*, *4CL*, and *CHS* had higher levels of constitutive expression (0 h) compared

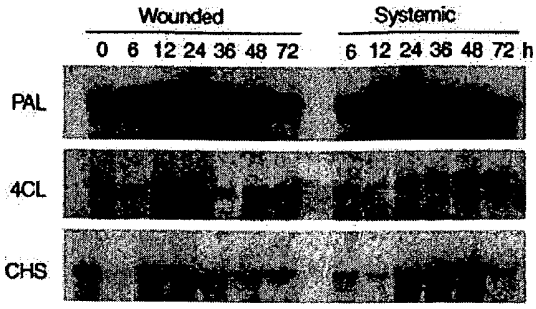

Figure 6. Northern analysis of key phenylpropanoid enzymes following simulated herbivory of aspen.
Previously hybridized membranes (Figure 4) were stripped and probed with phenylalanine ammonia-lyase (*PAL*), 4-coumarate:CoA ligase (*4CL*), and chalcone synthase (*CHS*). The *PAL* blot was exposed for 18 h, and the *CHS* and *4CL* blots were exposed for 24 h.

to *DFR*, likely a result of their role in the synthesis of other constitutive phenylpropanoids such as lignin or phenolic glycosides. We also found a decrease in *CHS* and *4CL* mRNA at 6 h in wounded tissue, and at 12 h in systemically induced tissue (Figure 6). This decrease was not expected yet repeatedly observed; it might be related to circadian regulation of phenylpropanoid metabolism in *Arabidopsis*, as described (Harmer *et al.*, 2000). Overall, these experiments indicate the upregulation of other phenylpropanoid enzymes in parallel with DFR induction.

Further characterization of DFR *expression*

We next investigated *DFR* expression following feeding by two leaf-eating aspen pests, forest tent caterpillar (FTC) and satin moth (*Leucoma salicis*). Leaves of LPI 12–17 of 3-month-old aspen plants were damaged by FTC as described in the Experimental procedures, which resulted in removal of approximately 10–25% of leaf area. The damaged leaves, as well as undamaged (systemic) leaves of LPI 7–10, were harvested after 24 h and analyzed by Northern hybridization. Control samples were harvested from identical trees which were placed in a cage without any larvae. *DFR* mRNA was present only at low levels in leaves of control plants (Figure 7a, C). However, in trees damaged by FTC larvae, *DFR* mRNA was induced to high levels in all leaves, both wounded and unwounded (Figure 7a, W). The degree of *DFR* mRNA induction by FTC damage was comparable to that induced by mechanical wounding. Strong *DFR* induction was also observed in experiments with satin moth larvae. In this experiment, the larvae were allowed to freely move and feed on an entire aspen plant for 96 h. The caterpillar larvae showed a preference for older leaf tissue (LPI 8–12), as many of the young leaves remained uneaten (LPI 2–5). Leaves of several age classes were harvested for Northern blot analysis, and *DFR* mRNA expression was found to be induced to high concentrations throughout

the plant (Figure 7b). Continuous feeding by satin moth larvae induced *DFR* expression to higher levels than those seen in the FTC and mechanical wounding experiments. These experiments demonstrate that aspen *DFR* mRNA expression is induced by both aspen herbivores, and that this induction is systemic. This suggests that *DFR* expression is part of the induced defense response against insects.

We also tested the effects of MeJa, a well-known inducer of plant defense responses (Weiler, 1997). We had previously shown that this signal compound induces trypsin inhibitor and *PPO* genes, both components of the protein-based aspen defense. *DFR* expression was clearly induced by MeJa in both young and old plants (Figure 7c), showing levels that are comparable to mechanically wounded aspen leaves. Young leaves showed a stronger induction of *DFR* compared to old leaves, as was observed for other defense genes (Haruta *et al.*, 2001a,b).

Since CTs are also produced constitutively in both leaf and woody tissues of trembling aspen (Lindroth and Hwang, 1996), we analyzed the constitutive expression of *DFR* in several aspen tissues. *DFR* mRNA expression was expressed at low levels in healthy leaves, petioles, stems, and roots (Figure 7c). However, based on relative signal intensities in Northern blots probed simultaneously, the levels of *DFR* mRNA in control tissues were about 7–10 times lower than those found in wounded leaves. The *n*-butanol:HCl assays indicated that significant levels of CTs were present in these tissues, with the exception of petioles; however, these contained high levels of anthocyanins (data not shown). In addition, *DFR* mRNA expression was demonstrated in female but not male flowers of outdoor grown aspen trees. We conclude that *DFR* is constitutively expressed at low levels throughout trembling aspen saplings, and that these expression levels generally correlate with CTs.

To determine if the observed pattern of DFR induction is specific to trembling aspen, we compared wound and FTC induction of *DFR* in aspen to that in hybrid poplar (*P. trichocarpa* × *deltoides*). Hybrid poplar has a very strong inducible defense that includes both trypsin inhibitors and PPO (Constabel *et al.*, 2000). Surprisingly, compared to aspen, DFR induction in hybrid poplar was very low (Figure 7d, top panel). When the blot was reprobed with PPO, strong signals in both samples confirmed that hybrid poplar was responding strongly to both types of damage (Figure 7d, bottom panel). Therefore, it appears that strong DFR induction is characteristic of *P. tremuloides*, but may not be found in other *Populus* species.

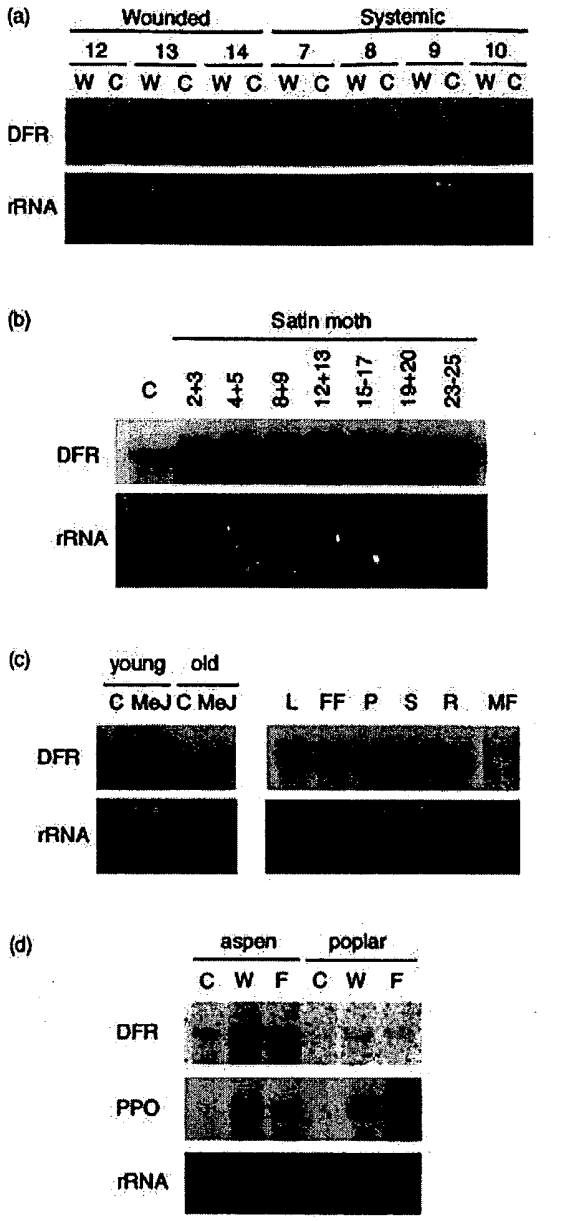

Figure 7. Northern analysis of *DFR* expression in leaves following herbivory or MeJa treatment and in undamaged tissues.
Total RNA was extracted and analyzed by RNA blot as described above.
(a) FTC larvae fed on leaves of LPI 12–14, and the damaged and undamaged leaves (LPI 7–10; systemic) on wounded (W) trees were harvested 24 h after feeding. Identical leaves LPI (7–14) from an unwounded plant were used as a control (C).
(b) Satin moth larvae fed continuously on aspen plants for 96 h, and leaves were grouped by age categories for analysis.
(c) Left panel: Young (4-week-old) and old (12-week-old) plants were treated with methyl jasmonate (MeJa) for 24 h, or mock-sprayed (C). Right panel: Developmental expression of *DFR* in unwounded plants. L, leaves; P, petioles; S, stems; and R, roots. FF and MF refer to male and female flowers from wild trees, respectively.
(d) Comparative analysis of DFR and PPO induction in aspen and poplar. Plants were induced for 24 h (C, control; W, wounded; and F, FTC damaged.) All blots were exposed for 24 h. The ethidium bromide-stained gels are shown as loading controls.

Discussion

The accumulation of CTs following herbivore attack has been observed in a number of tree species, but the under-

lying molecular biology of this induction is not known. Here, we have investigated this response by cloning a cDNA encoding a key enzyme of CT biosynthesis, DFR, and characterizing its expression. We demonstrate that DFR is induced by herbivory and other treatments known to trigger the herbivore defense response in aspen. We also show that subsequent to *DFR* mRNA induction, both DFR activity and CTs increase, suggesting that the observed *DFR* mRNA induction is relevant for aspen defense.

We used PCR and available DFR sequence data to obtain a probe which was used to isolate a full-length *DFR* cDNA. Detailed sequence analysis of *PtDFR* predicted a DFR protein of 38.8 kDa with a typical NADPH-binding domain (Figure 2). Analysis by PSORT predicted a membrane association for the DFR protein; this would be consistent with the observation that CHS, chalcone isomerase (CHI), and DFR associate as an enzyme complex (Burbulis and Winkel-Shirley, 1999). CHS and CHI have been found to be localized at the endoplasmic reticulum and vacuolar membranes (Saslowsky and Winkel-Shirley, 2001). Inspection of the substrate binding domain revealed that residue 134, a conserved asn residue in most DFR proteins, has been replaced by an asp in aspen DFR. This position is known to be important for substrate specificity, since site-directed mutagenesis of asn-134 to leu in *Gerbera* DFR results in an enzyme with altered substrate preferences (Johnson *et al.*, 2001). *Petunia* DFR, like aspen DFR, also contains an asp residue at position 134, and as a result is unable to reduce dihydrokaempferol (4′ hydroxylated on the B-ring) (Johnson *et al.*, 2001). This suggests that aspen DFR should also have a high affinity for dihydroquercetin and dihydromyricetin (3′,4′ and 3′,4′,5′ hydroxylated on the B ring, respectively), but not for dihydrokaempferol. This hypothesis could be tested using our recombinant DFR. A preference for the more highly hydroxylated dihydroflavonol substrates, dihydroquercetin and dihydromyricetin, would be consistent with an antiherbivore role of *PtDFR* and aspen CT structure. Greater hydroxylation is predicted to allow for greater hydrogen bonding, one of the mechanisms leading to the formation of tannin–protein complexes (Hagerman and Butler, 1989). When comparing CTs from a number of different species, Ayres *et al.* (1997) found a general correlation of higher antiherbivore activity with a greater proportion of prodelphinidin (trihydroxylated) units. Aspen leaf CT has a relatively high prodelphinidin:procyanidin ratio, 57:43 (Ayres *et al.*, 1997).

Southern analysis at high stringency detected only one major hybridizing band in aspen genomic DNA, suggesting that the aspen genome contains a single copy of *DFR* (Figure 3a). By contrast, Southern blots performed with hybrid poplar (*P. trichocarpa* × *deltoides*) genomic DNA showed two *DFR* bands (not shown); these most likely represent the two distinct parental *DFR* alleles. Altogether, our results are most consistent with a single *DFR* locus being present in *Populus*. The presence of fainter bands seen at less stringent washing conditions suggested the presence of additional *DFR*-like genes in the aspen genome, perhaps encoding enzymes with related functions (Figure 3b). Specifically, in *Arabidopsis*, the *DFR*-like *BAN* gene appears to encode leucoanthocyanidin reductase, which converts leucoanthocyanidin to catechin (Devic *et al.*, 1999). This enzyme acts immediately downstream of DFR in CT biosynthesis (Figure 1), and thus a *DFR*-like leucoanthocyanidin reductase gene is also expected to be present in the aspen genome. Experiments are under way to isolate this gene from aspen.

Northern analysis suggested that in aspen leaves, *DFR* expression is induced by real and simulated herbivory (Figures 4 and 7), the first such report for any plant. Although it is becoming increasingly clear that many plants respond differently to mechanical wounds and insect herbivory (Walling, 2000), we found no obvious indication of this at the level of *DFR* expression. The observed *DFR* mRNA induction is very likely to be important for herbivore defense, since it is followed first by increased DFR enzyme activity and later by a higher concentration of the CT itself. DFR activity and CT accumulation were induced only approximately twofold in our experiments, compared to an increase in *DFR* mRNA levels of 7–10-fold. However, *PAL*, *4CL* and *CHS* all appeared to be induced by wounding in parallel to *DFR* (Figure 6), suggesting that wounding upregulates the general phenylpropanoid pathway and confirming that phenylpropanoid biosynthesis is stimulated. Wound induction of *PAL* and *4CL* has been observed in many plants (Hahlbrock and Scheel, 1989), and *CHS* was recently shown to be inducible in white spruce (Richard *et al.*, 2000). Despite this observed upregulation of phenylpropanoid genes, however, the accumulated products are not always known.

In our system, we have consistently observed a wound induction of CT, as has been described previously (Osier and Lindroth, 2001). We used two different assay methods to measure CT, one based on structural features (*n*-butanol–HCl) and the other based on function (radial diffusion/protein precipitation assay). Both assays gave very similar results (Figure 5). The BSA radial diffusion assay measures precipitation of protein *in vitro*; since tannins are detrimental to many insect pests at least in part due to their protein-precipitating ability, it is likely that wounded aspen leaves will be a lower quality food source than control leaves. This is consistent with the deleterious effects of aspen CTs on lepidopteran larvae (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997). It is important to point out that we used sainfoin rather than aspen CT as a standard, and these may give slightly different responses in our assays. The absolute values obtained can therefore be considered as estimates only. However, this should not affect the validity of the

relative differences between samples and treatments we have observed. We also note that for a given tannin, protein-precipitating ability depends very much on the protein being assayed, so that BSA may give a different response than aspen leaf protein (Giner-Chavez *et al.*, 1997). Again, this does not limit the usefulness of the assay to detect relative changes in protein-precipitating ability.

The importance of DFR in aspen defense was confirmed by the co-ordinate induction of other defense proteins, PPO (Figure 4) and trypsin inhibitor (TI; Haruta *et al.*, 2001a). PPO can act as an antinutritive defense via production of reactive quinones which alkylate dietary protein (Duffey and Felton, 1991). TIs interfere with digestive functions in insects, and purified aspen TI showed a negative impact on the growth of test insects (Haruta *et al.*, 2001a; N. Hotte and C.P. Constabel, unpublished data). Our work demonstrates that like *PPO* and *TI* expression, herbivore- and wound-induced *DFR* and CT accumulation in aspen is a systemic response. The responsiveness of *DFR* to MeJa suggests that the signal transduction pathway includes endogenous jasmonate signals (Weiler, 1997). Taken together, the similarity with well-characterized signaling paths in tomato (Bergey *et al.*, 1996) suggests that CT accumulation in response to aspen herbivory is likely mediated by an octadecanoid signal transduction mechanism. To our knowledge, defense-related *DFR* expression and CT accumulation have not been extensively investigated in this context; CT induction by jasmonates was only recently documented (Arnold and Schultz, 2002), and one prior study reported DFR induction by wounding and MeJa, leading to anthocyanin synthesis in *Petunia* corollas. (Tamari *et al.*, 1995).

The phenomenon of tannin and phenolic induction by real or simulated herbivory has generally been investigated in field experiments, with high variability within and among plants (Hartley and Lawton, 1991). We also observed substantial variability in CT levels between individual plants, even in growth rooms. However, by correlating CT levels with *DFR* expression, we were able to use the *PtDFR* cDNA as a sensitive probe in Northern analyses, which showed much less plant-to-plant variation. Therefore, we now have strong evidence demonstrating that induced CT accumulation is a component of the systemic response in aspen. This suggests that in aspen, phytochemical and protein-based defenses are induced by the same signaling system, and future experiments will test this hypothesis.

That *DFR* has not previously been identified as an herbivore-inducible defense gene in other plants is likely due to the specialization of defensive chemistry in the plant kingdom. Tannins are typical phytochemicals of woody plants and generally not found in herbs (Swain, 1979), so clearly not all plant species use CTs as part of their defense arsenal. Furthermore, among woody plants, herbivore induction of tannins has been reported only some species, for example in birch, oak, willow, and poplar (reviewed in Arnold and Schultz, 2002; reviewed in Constabel, 1999). There is also differentiation and specialization within *Populus*, since under identical treatments, hybrid poplar (*P. trichocarpa* × *deltoides*) showed only weak *DFR* mRNA induction (Figure 7d) and little CT accumulation (data not shown). Interestingly, the same poplar hybrid exhibits a very strong induced defense response, as seen by the increase in PPO, TI, and other defense proteins (Figure 7d; Bradshaw *et al.*, 1989; Constabel *et al.*, 2000). A different poplar hybrid (*P. deltoides* × *nigra*) does show jasmonic acid induction of CTs; however, this appears to be restricted to very young (LPI 3) leaves (Arnold and Schultz, 2002). We speculate that hybrid poplar relies more on protein-based defense, while aspen has evolved to induce phytochemical defenses as well. This underscores the importance of studying plant defense in a variety of experimental systems. It will be interesting to compare defense strategies in other *Populus* species, and to explore the molecular basis for the variation in herbivore resistance seen in natural populations in the field.

Experimental procedures

Plant growth conditions and stress treatments

Trembling aspen (*P. tremuloides*) were micropropagated and maintained in the University of Alberta Biotron's environmental chambers as described (Haruta *et al.*, 2001a). Trees used in our experiments had at least 25 leaves, which were identified using the Leaf Plastochron Index (LPI; Larson and Isebrands, 1971). The index leaf (LPI 0) was determined to be the first developing leaf with a lamina length of 20 mm. For routine wounding experiments, leaves of LPI 12–15 were designated as systemic leaves, and leaves of LPI 16–19 were wounded. Mechanical wounding to simulate herbivory was performed by crushing the leaf margins using a hemostat three times at 2-h intervals. Time course experiments for phytochemical assays involved wounding six leaves on each of eight trees, with both wounded and unwounded upper (systemic) leaves being harvested at the appropriate times. Sampling at each time point involved arbitrarily selecting five trees from among the eight wounded trees, and sampling one wounded leaf from each tree. Systemic leaves were chosen by the same system. The collection of leaves was performed in such a way that no tree would lose a disproportionate number of leaves to the sampling procedure.

Time course and wounding experiments were started at approximately 9 AM. Insect experiments were conducted in 75-cm high insect cages placed within growth chambers with locally collected insect larvae. For FTC herbivore experiments, 12 fifth instar forest tent caterpillar (*Malacosoma disstria*) larvae were placed on each tree and allowed to feed for three 20-min intervals every 2 h. Satin moth (*Leucoma salicis*) experiments were conducted with 20 fourth and fifth instar larvae which fed continuously for 96 h on plants within insect cages. Plants were treated with MeJa as previously described (Haruta *et al.*, 2001a). All harvested plant tissues were frozen in liquid nitrogen and stored at −80°C until analyzed.

Isolation of a DFR *cDNA and expression in* E. coli

To isolate a *DFR* fragment from a trembling aspen cDNA library, degenerate primers were designed from an amino acid sequence alignment of DFR enzymes from several plants in the GenBank database. PCR was performed with 5′ primer DFR 2-D (5′-GAT/C CCN A/GAA/G AAT/C GAA/G A/GTN ATT/C/A AAA/G CC-3′) and 3′ primer DFR 3-U (5′-A/GAA A/GTA CAT CCA NG/CC NGT CAT C/TTT-3′) with an aspen cDNA library as template using 20 cycles of 94°C (60 sec)/49°C (60 sec)/72°C (30 sec) followed by 20 cycles of 94°C (60 sec)/51°C (60 sec)/72°C (30 sec). A PCR product of predicted size was extracted from agarose gels (QIAEX II, Qiagen), cloned into pBluescript T-vectors (Marchuk *et al.*, 1991) and sequenced. The aspen cDNA library was screened using standard molecular techniques (Sambrook *et al.*, 1989). DNA sequencing of *DFR* cDNAs was performed on both strands using both the Applied Biosystems 377 Automatic Sequencer, or the Beckman CEQ Automatic Sequencer. DNA sequences were analyzed using Sequencher 4.1.2 (Gene Codes Corporation), PSORT (http://psort.nibb.ac.jp), and ExPASy (http://c.expasy.org) programs.

For bacterial overexpression, the coding sequence of *DFR* was PCR amplified from the full-length *DFR* plasmid clone (*pDFR14a*) using 5′ primer DFRUPPER (5′-GGG CGG ATC CAA GAT GGG AAC AGA AGC-3′) and 3′ primer DFRLOWER (5′-GGG GAA GCT TGA GGA ACA ATC AGG ACG-3′) and cloned into the pQE30 bacterial expression vector (Qiagen). The resulting plasmid was moved into *E. coli* strain M15(pREP4). Cultures were grown at 30°C overnight in the presence of 1 mM IPTG, pelleted by centrifugation, and resuspended in 0.1 M Tris–HCl (pH 7.4) containing 20 mM sodium ascorbate. Cells were disrupted using sonication, the suspension centrifuged, and the supernatant used directly for DFR activity assays (see below).

Amplification of phenylpropanoid gene fragments

PCR primers for *PAL* and *4CL* were designed from published *Populus* sequences (Hu *et al.*, 1998; Subramaniam *et al.*, 1993), and *CHS* primers were based on consensus sequence information from several plant species in public databases. Primers used to clone the respective genes are as follows: PALS1, 5′-GGC GTT ACT GGT TTT GGT GC-3′; PALA1, 5′-TTG AAG CCA TAA TCC AAA CTT GG-3′; 4CLS1, 5′-AGT GTG GCT CAA CAA/G GTA/T GAT GG-3′; 4CLA1, 5′-AAC/T GCA/G ACA GGA ACT TCA/T CC-3′; CHSS1, 5′-GCN ATA/T/C AAA/G GAA/G TGG GGN CAA/G CC-3′; CHSA1′ 5′-GGN CCN CCN GGA/G TGN GCA/T/G ATC C-3′. PCR amplification from aspen cDNA library was performed with 34 cycles of 95°C (30 sec)/54°C (45 sec)/72°C (60 sec). DNA products were cloned and sequences were determined as described above.

DNA and RNA hybridization analysis

All molecular techniques followed standard protocols (Sambrook *et al.*, 1989). Genomic DNA was isolated from trembling aspen using the DNEasy Maxi Plant Kit (Qiagen), and 10 μg was digested for 16 h with 50 U of *Hin*dIII, *Nco*I, *Xba*I, and *Eco*RI (Gibco/BRL). Southern analysis was performed with a full-length ^{32}P-labelled DFR clone. Membrane filters were washed twice at 65°C in 1X SSPE, 0.1% SDS for 30 and 10 min (low stringency), and again at 65°C in 0.1X SSPE, 0.1% SDS for 30 and 10 min (high stringency). Hybridization signals were detected by autoradiography or by exposure on a Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA). Total RNA was extracted from 0.5 g of aspen tissues according to the RNA extraction protocol described in Haruta *et al.* (2001a). Northern analysis was performed by separating 15 μg of total RNA on 1.2% agarose–formaldehyde gels, blotting to Zeta-probe membranes (Bio-Rad), hybridizing, and washing at low stringency.

DFR enzyme extraction and assay

Approximately 1.5 g of frozen aspen tissue was extracted in 5 ml of 0.1 M borate–HCl (pH 8.8) buffer containing 20 mM sodium ascorbate, 10% (v/v) glycerol, 0.3 g dry polyvinylpolypyrrolidone (Sigma), and 0.5 g dry Amberlite XAD-4 (Sigma). Samples were centrifuged and the supernatant fraction de-salted through a medium resin Sephadex G-25 PD-10 desalting column (Pharmacia) equilibrated with 0.1 M Tris–HCl (pH 7.4) and 20 mM sodium ascorbate. Protein concentrations were determined by the Bradford method (Bradford, 1976) using BSA as a standard. DFR activity was measured according to the assay described by Stafford and Lester (1982). Reactions contained 0.1 M Tris–HCl (pH 7.4), 1 mM dihydroquercetin (+/–taxifolin, Sigma), 1 mM NADPH and 0.3 ml enzyme extract in a 1-ml volume. A NADPH-regenerating system consisting of 1 U glucose-6-phosphate dehydrogenase and 6 μM glucose-6-phosphate was also added to the mixture. Reactions were incubated at 30°C for 30 min and stopped with the addition of 6 N HCl. Leucoanthocyanidins were extracted three times with ethyl acetate, which was then evaporated under vaccum. One milliliter of *n*-butanol:HCl (95:5 v/v) was added to the residue, and the A_{550} measured. Absorbance values were converted to nanomoles of cyanidin using 34 700 as a molar extinction coefficient (Stafford and Lester, 1982).

Phytochemical assays

Condensed tannin (proanthocyanidin) concentrations were determined using the *n*-butanol:HCl method as described by Porter *et al.* (1986). Purified CT standards, isolated from mature leaves of sainfoin (*Onybrychis viciifolia*), were obtained from M. Gruber, Agriculture and Agri-Food Canada, Saskatoon, Canada. These had been previously determined to have a degree of polymerization of 6–7, a prodelphinidin:procyanidin ratio of 88:12, and a *cis*:*trans* ratio of 67:33 (Koupai-Abyazani *et al.*, 1993). Protein precipitation by tannins in aspen leaf extracts was assayed by the radial diffusion method (Hagerman, 1987). One milliliter of proanthocyanidin extract was evaporated under vacuum to approximately 50 μl, and 8 μl added in several aliquots to a 4.0-mm-diameter well in the BSA plate. Plates were incubated at 30°C for 96 h, and diameters of the precipitation rings were measured using a caliper. Anthocyanins were extracted from aspen leaf tissue (100 mg) into 10 ml of 1% HCl in methanol for 24 h at 4°C, and A_{530} and A_{657} measured spectrophotometrically as described (Hasegawa *et al.*, 2001). Statistical analysis (two-way ANOVA) for phytochemical data was carried out using the SAS System for Windows v. 8.0 (SAS Institute, Cary, NC). Differences among mens were tested using Fisher's Least Significant Difference (LSD).

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References

Arnold, T.M. and Schultz, J.C. (2002) Induced sink strength as a prerequisite for induced tannin biosynthesis in developing leaves of *Populus*. *Oecologia*, **130**, 585–593.

Ayres, M.P., Clausen, T.P., MacLean,S.F., Jr, Redman, A.M. and Reichardt, P.B. (1997) Diversity of structure and antiherbivore activity in condensed tannins. *Ecology*, **78**, 1696–1712.

Barbehenn, R.V. and Martin, M.M. (1994) Tannin sensitivity of larvae of *Malacosoma disstria* (Lepidoptera): roles of the peritrophic envelope and midgut oxidation. *J. Chem. Ecol.* **8**, 1985–2001.

Bergey, D.R., Howe, G.A. and Ryan, C.A. (1996) Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. *Proc. Natl Acad. Sci. USA*, **93**, 12053–12058.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem.* **71**, 248–254.

Bradshaw, H.D., Jr, Hollick, J.B., Parsons, T.J., Clarke, H.R.G. and Gordon, M.P. (1989) Systemically wound-responsive genes in poplar trees encode proteins similar to sweet potato sporamins and legume Kunitz trypsin inhibitors. *Plant Mol. Biol.* **14**, 51–59.

Burbulis, I.E. and Winkel-Shirley, B. (1999) Interactions among enzymes of the *Arabidopsis* flavonoid biosynthetic pathway. *Proc. Natl Acad. Sci. USA*, **96**, 12929–12934.

Constabel, C.P. (1999) A survey of herbivore-inducible defensive proteins and phytochemicals. In *Induced Plant Defenses Against Herbivores and Pathogens* (Agrawaal, A.A., Tuzun, S. and Bent, E., eds). St. Paul: APS Press, pp. 137–166.

Constabel, C.P., Yip, L., Patton, J.J. and Christopher, M.E. (2000) Polyphenol oxidase from hybrid poplar. Cloning and expression in response to wounding and herbivory. *Plant Physiol.* **124**, 285–295.

Devic, M., Guilleminot, J., Debeaujon, I., Bechtold, N., Bensaude, E., Koornneef, M., Pelletier, G. and Delseny, M. (1999) The *BANYULS* gene encodes a DFR-like protein and is a marker of early seed coat development. *Plant J.* **19**, 387–398.

Dixon, R.A. and Paiva, N.L. (1995) Stress-induced phenylpropanoid metabolism. *Plant Cell*, **7**, 1085–1097.

Duffey, S.S. and Felton, G.W. (1991) Enzymatic antinutritive defenses of the tomato plant against insects. In *Naturally Occurring Pest Bioregulators* (Hedin, P.A., ed.). Washington, DC: ACS Press, pp. 167–197.

Giner-Chavez, B.I., Van Soest, P.J., Robertson, J.B., Lascano, C. and Pell, A.N. (1997) Comparison of the precipitation of alfalfa leaf protein and bovine serum albumin by tannins in the radial diffusion method. *J. Sci. Food Agric.* **74**, 513–523.

Gruppe, A., Fusseder, M. and Schopf, R. (1999) Short rotation plantations of aspen and balsam poplar on former arable land in Germany: defoliating insects and leaf constituents. *For. Ecol. Manag.* **121**, 113–122.

Hagerman, A.E. (1987) Radial diffusion method for determining tannin in plant extracts. *J. Chem. Ecol.* **13**, 437–449.

Hagerman, A.E. and Butler, L.G. (1989) Choosing appropriate methods and standards for assaying tannins. *J. Chem. Ecol.* **15**, 1795–1810.

Hagerman, A.E. and Butler, L.G. (1991) Tannins and lignins. In *Herbivores: Their Interaction with Secondary Plant Metabolites, Vol. I* (Rosenthal, G.A. and Berenbaum, M.R., eds). San Diego: Academic Press, pp. 355–387.

Hahlbrock, K. and Scheel, D. (1989) Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 347–369.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.-S., Han, B., Zhu, T., Wang, X., Kreps, J.A. and Kay, S.A. (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science*, **290**, 2110–2113.

Hartley, S.E. and Lawton, J.H. (1991) Biochemical aspects and significance of the rapidly induced accumulation of phenolics in birch foliage. In *Phytochemical Induction by Herbivores* (Tallamy, D.W. and Raupp, M.J., eds). New York: John Wiley, pp. 105–132.

Haruta, M., Major, I.T., Christopher, M.E., Patton, J.J. and Constabel, C.P. (2001a) A Kunitz trypsin inhibitor gene family from trembling aspen (*Populus tremuloides* Michx.): cloning, functional expression, and induction by wounding and herbivory. *Plant Mol. Biol.* **46**, 347–359.

Haruta, M., Pedersen, J.A. and Constabel, C.P. (2001b) Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, and potential substrates. *Physiol. Plant.* **112**, 552–558.

Hasegawa, H., Fukasawa-Akada, T., Okuno, T., Niizeki, M. and Suzuki, M. (2001) Anthocyanin accumulation and related gene expression in Japanese parsley (*Oenanthe stolonifera*, DC.) induced by low temperature. *J. Plant Physiol.* **158**, 71–78.

Haslam, E. (1993) Polyphenols: phytochemical chameleons. In *Phytochemistry and Agriculture* (van Beek, T.A. and Breteler, H., eds). Oxford: Clarendon Press, pp. 215–252.

Hemming, J.D.C. and Lindroth, R.L. (1995) Intraspecific variation in aspen phytochemistry: effects on performance of gypsy moths and forest tent caterpillars. *Oecologia*, **103**, 79–88.

Hu, W.-J., Kawaoka, A., Tsai, C.-J., Lung, J., Osakabe, K., Ebinuma, H. and Chiang, V.L. (1998) Compartmentalized expression of two structurally and functionally distinct 4-coumarate: CoA ligase genes in aspen (*Populus tremuloides*). *Proc. Natl Acad. Sci. USA*, **95**, 5407–5412.

Hwang, S.-Y. and Lindroth, R.L. (1997) Clonal variation in foliar chemistry of aspen: effects on gypsy moths and forest tent caterpillars. *Oecologia*, **111**, 99–108.

Ives, W.G.H. and Wong, H.R. (1988) *Tree and Shrub Insects of the Prairie Provinces*. Information report NOR-X-292. Edmonton: Canadian Forestry Service.

Johnson, E.T., Ryu, S., Yi, H., Shin, B., Cheong, H. and Choi, G. (2001) Alteration of a single amino acid changes the substrate specificity of dihydroflavonol 4-reductase. *Plant J.* **25**, 325–333.

Karban, R. and Baldwin, I.T. (1997) *Induced Reponses to Herbivory*. Chicago: The University of Chicago Press.

Koupai-Abyazani, M.R., McCallum, J., Muir, A.D., Bohm, B.A., Towers, G.H.N. and Gruber, M.Y. (1993) Developmental changes in the composition of proanthocyanidins from leaves of sainfoin (*Onobrychis viciifolia* Scop.) as determined by HPLC analysis. *J. Agric. Food Chem.* **41**, 1066–1070.

Lacombe, E., Hawkins, S., Doorsselaere, J.V., Piquemal, J., Goffner, D., Poeydomenge, O., Boudet, A.-M. and Grima-Pattenati, J. (1997) Cinnamoyl CoA reductase, the first committed enzyme of the lignin branch biosynthetic pathway: cloning, expression and phylogenetic relationships. *Plant J.* **11**, 429–441.

Larson, P.R. and Isebrands, J.G. (1971) The plastochron index as applied to developmental studies of cottonwood. *Can. J. For. Res.* **1**, 1–11.

Lindroth, R.L. and Hwang, S.-Y. (1996) Diversity, redundancy, and multiplicity in chemical defense systems of aspen. In

Phytochemical Diversity and Redundancy in Ecological Interactions (Romeo, J.T., Saunders, J.A. and Barbosa, P., eds). New York: Plenum Press, pp. 25–56.

Marchuk, D., Drumm, M., Saulino, A. and Collins, F.S. (1991) Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. *Nucl. Acids Res.* **19**, 1154.

Olsen, O., Wang, X. and von Wettstein, D. (1993) Sodium azide mutagenesis: preferential generation of AT → GC transitions in the barley *Ant18* gene. *Proc. Natl Acad. Sci. USA*, **90**, 8043–8047.

Osier, T.L. and Lindroth, L.R. (2001) Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. *J. Chem. Ecol.* **27**, 1289–1313.

Porter, L.J., Hrstich, L.N. and Chan, B.G. (1986) The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry*, **25**, 223–230.

Richard, S., Lapointe, G., Rutledge, R.G. and Seguin, A. (2000) Induction of chalcone synthase expression in white spruce by wounding and jasmonate. *Plant Cell Physiol.* **41**, 982–987.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Saslowsky, D. and Winkel-Shirley, B. (2001) Localization of flavonoid enzymes in *Arabidopsis* roots. *Plant J.* **27**, 37–48.

Schultz, J.C. and Baldwin, I.T. (1982) Oak leaf quality declines in response to defoliation by gypsy moth larvae. *Science*, **217**, 149–151.

Shirley, B.W., Kubasek, W.L., Storz, G., Bruggemann, E., Koornneef, M., Ausubel, F.M. and Goodman, H.M. (1995) Analysis of *Arabidopsis* mutants deficient in flavonoid biosynthesis. *Plant J.* **8**, 659–671.

Stafford, H.A. and Lester, H.H. (1982) Enzymic and non-enzymic reduction of (+)-dihydroquercitin to its 3,4-diol. *Plant Physiol.* **70**, 695–698.

Subramaniam, R., Reinold, S., Molitor, E.K. and Douglas, C.J. (1993) Structure, inheritance, and expression of hybrid poplar (*Populus trichocarpa* × *Populus deltoides*) phenylalanine ammonia-lyase genes. *Plant Physiol.* **102**, 73–83.

Swain, T. (1979) Tannins and lignins. In *Herbivores: Their Interaction with Secondary Plant Metabolites* (Rosenthal, G.A. and Janzen, D.H., eds). San Diego: Academic Press, pp. 657–682.

Tamari, G., Borochov, A., Atzorn, R. and Weiss, D. (1995) Methyl jasmonate induces pigmentation and flavonoid gene expression in petunia corollas: a possible role in wound response. *Physiol. Plant.* **94**, 45–50.

Walling, L.L. (2000) The myriad plant responses to herbivores. *J. Plant Growth Regul.* **19**, 195–216.

Weiler, E.W. (1997) Octadecanoid mediated signal transduction in higher plants. *Naturwissenschaften*, **84**, 340–349.

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CHEMICAL REACTION DETECTION OF CATECHINS AND PROANTHOCYANIDINS WITH 4-DIMETHYLAMINOCINNAMALDEHYDE

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SUMMARY

An high-performance liquid chromatographic method with post-column derivatization is described which allows the specific detection of catechins and proanthocyanidins in crude extracts from plants and beverages. In the presence of concentrated sulphuric acid, 4-dimethylaminocinnamaldehyde can be employed as a selective reagent. The advantage of the reagent is that its condensation products with flavanols show maximum absorbance at about 640 nm. Other phenols, indoles and terpenes give reaction products with different absorbances or react very weakly. A 200–40 000 fold sensitivity was found for (−)-epicatechin as compared to other phenols and substituted indoles. Concerning the terpenes, this factor ranges from 4000 (for the aromatic thymol) to $2 \cdot 10^6$.

INTRODUCTION

Catechins and their oligomeric forms, namely the proanthocyanidins (condensed tannins), are an heterogeneous group of secondary compounds[1,2] which are widespread in the plant kingdom[3–5]. The astringency of these flavanols is well known in fruits[6–8]. The content of catechins is an important factor in determining the quality of juices[9] and wines[10–12]. The oligomeric proanthocyanidins play a rôle in the durability of beers[13–15]. All the above mentioned observations are related to the ability of flavanols to precipitate proteins[16]. This precipitation reaction is probably also responsible for the participation of catechins in plant defence mechanisms[17,18].

For these reasons it is often necessary to know the composition of the various catechins and condensed tannins in plant tissues and to monitor their structural variation during beverage processing[19–23] or during the wound response of plants[24,25]. In the latter case, oxidation processes often lead to oligomerization and polymerization[1] and diseased plant tissues sometimes show an enhanced synthesis of flavanols[26].

The analytical method normally used to estimate the amount of catechin and its derivatives is the colorimetric measurement of their total content after reaction with aromatic aldehydes[27–29] in a test-tube. The qualitative pattern of these phenols can be determined by thin-layer chromatography using the known aromatic aldehydes[30–32]

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or the trinitrophenol–potassium hydroxide reagent[33] for visualization. However, the quantification of each compound normally requires its purification from other phenolic compounds. A prepurification has been carried out by several authors[34,35]. In high-performance liquid chromatographic (HPLC) analysis of flavanols extracted from plants, which are rich in phenols, the main problem is the rather low molar extinction of catechins as compared to phenolic acids. The cinnamic acids also show similar retention behaviours and often occur in plants in a more concentrated form than the flavanols. Lea[36] solved this problem using a pH-shift technique during the HPLC separation of apple juices. A chemical reaction detection of flavanols by using 4-dimethylaminocinnamaldehyde (DMACA) after their preparative separation on a Sephadex column has been described by McMurrough and McDowell[37] and McMurrough[38].

This paper deals with the post-column derivatization of catechins and proanthocyanidins for their selective detection following analytical HPLC separation of crude plant extracts and beverages.

EXPERIMENTAL

The HPLC equipment consisted of two pumps T-414 (Kontron) and the gradient programmer 205 (Kontron). The column (250 mm × 4 mm I.D.) was prepacked with Shandon Hypersil ODS, 3 μm. The solvents were 5% acetic acid (A) and methanol (B).

Gradient range: 0–5 min, isocratic, 5% B in A; 5–10 min, 5–10% B in A; 15–25 min, 10–15% B in A; 25–35 min, isocratic, 15% B in A; 35–37 min, 15–20% B in A; 37–45 min, isocratic, 20% B in A; 45–55 min, 20–30% B in A; 55–70 min, 30–45% B in A; 70–90 min, 45–90% B in A.

Because of the corrosive reagent, an inert HPLC pump (Gynkotek, F.R.G.) was used. It was equipped with titanium pump heads. Capillaries and screws were both made of PTFE. The reactor was a knitted PTFE capillary (9 m × 0.5 mm I.D.) as described by Engelhardt and Klinkner[39]. The substrate–reagent mixing was performed by a simple T-connection (titanium). The compounds were detected with an inert UV–VIS detector (Gynkotek, F.R.G.).

The estimation of the absorbance maximum and the wavelength ratio (640:620 nm) was performed with a Beckman Model 24 spectrophotometer.

RESULTS AND DISCUSSION

Application possibilities of aldehyde reagents

The reactivity of aldehydes in solutions containing strong mineral acids as well as the colour reactions of aromatic aldehyde have long been used to detect many different substances. Unsaturated compounds such as phenols[40–46], pyrroles and indoles[41,44] as well as some terpenes[42,43,45,47,48] were reported to react with aldehydes. Additionally, aliphatics, *i.e.*, alcohols, ketones may be converted into olefins under the influence of mineral acids and may then be sensitive to the aldehyde reaction[43,47,49].

In spite of these findings, colour reactions of aromatic aldehydes have often been employed specifically, such as for flavanols[29,38,45,50–57] or for indoles[58–61].

TABLE I
ABSORBANCE OF THE CONDENSATION PRODUCTS OF DMACA WITH (−)-EPICATECHIN IN THE PRESENCE OF VARIOUS ALCOHOLS
Concentration of sulphuric acid was 1.5 *M* in the corresponding alcohol.

Alcohol	*Wavelength of maximum absorbance (nm)*
Methanol	632
Ethanol	636
Propanol	638
Butanol	640

Mode of action

Principally, when dissolved in strong acids, aldehydes become electrophilic and therefore very reactive. The reaction mechanism with formaldehyde and phenols has been clarified by Finn and James[62] and by Hillis and Urbach[63]. However, such aromatic aldehydes, which are substituted, show a reduced reactivity, as compared to formaldehyde, because of the possible delocalization of the positive charge[64,65]. This requires an activated aromatic ring of the substrate, *i.e.*, of the phloroglucinol type in order to obtain optimum condensation reaction with phenols.

DMACA has the advantage that its reaction product with catechin shows an absorbance maximum between 632 and 640 nm depending on the solvent (Table I). Other aldehydes, commonly used, lead to absorption at a shorter wavelength (Table II), so that anthocyanidins or other substances which yield a red colour in the presence of acid may interfere. Moreover, the molar extinction of the products yielded with DMACA is about 1000 times higher than that with 4-dimethylaminobenzaldehyde[51].

Optimization of the derivatization system

As shown by several authors[51,60], the reaction with DMACA depends on the concentration of acid and alcohol. Since in the HPLC separation of flavanols a gradient system is necessary, the reaction conditions change during an experiment. Methanol both accelerates the reaction and increases the extinction value. However, after reaching the maximum absorbance, the extinction declined, which may be

TABLE II
ABSORBANCE OF THE CONDENSATION PRODUCTS OF VARIOUS ALDEHYDES WITH (−)-EPICATECHIN IN THE PRESENCE OF 0.075 *M* SULPHURIC ACID IN METHANOL

Aldehyde	*Wavelength of maximum absorbance (nm)*
Anisaldehyde	455
Vanillin	490
4-Dimethylaminobenzaldehyde	510
Syringaldehyde	515
4-Dimethylaminocinnamaldehyde	632

explained by a superimposed decomposition of the condensation product. The latter is also influenced by the acid and the alcohol concentration (Fig. 1). Water, acetonitrile and acetone inhibit the formation of the coloured product (Fig. 2).

In order to obtain a good sensitivity, 1% DMACA in 1.5 *M* methanolic sulphuric acid was used. The length of the knitted capillary reactor was 9 m, resulting in a reaction time of 90 s.

Selectivity and sensitivity

The use of the DMACA reagent for the specific detection of catechins demands knowledge of the relative sensitivity of other substances which are also known to give coloured products with aromatic aldehydes.

For this purpose the method of flow injection analysis[39] was used. Except for omission of the column, the system was the same as that described for the separation procedure. The solvent normally consisted of 40% methanol in 1% aqueous acetic acid. Only for some phenols and terpenes, butanol–methanol (1:5, v/v) was used as the solvent and the reagent was dissolved in butanol (containing 1.5 *M* sulphuric acid) to prevent demixing. For each compound a calibration graph was constructed to estimate the relative sensitivity.

Possible interference with catechins was shown to depend on the activation of the phenol group which determines the sensitivity towards the reagent. For this reason,

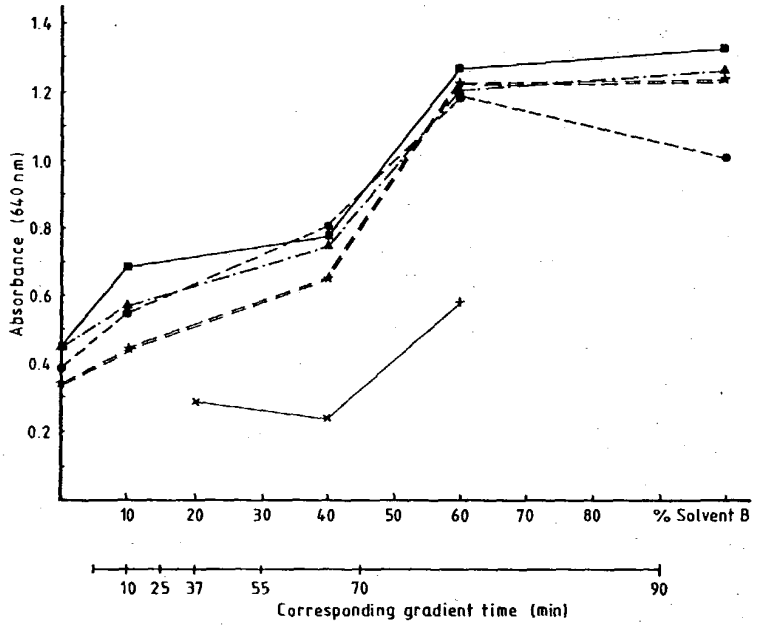

Fig. 1. Influence of the solvent composition and the reaction conditions (concentration of sulphuric acid in the 1% DMACA reagent; reaction time) on the detection sensitivity (640 nm) for (−)-epicatechin. Each data point represents the mean of four injections during flow injection analysis without the column. The injected flavanol was dissolved in that solvent which corresponded to the flow condition used at each point. (■) 0.65 *M*, 90 s; (▲) 1.15 *M*, 120 s; (●) 1.65 *M*, 60 s; (★) 5.30 *M*, 90 s; (×) 2.55 *M*, 90 s.

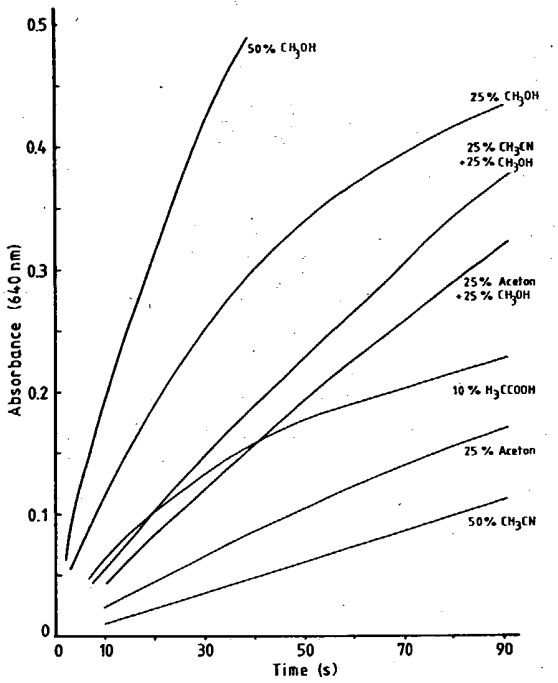

Fig. 2. Influence of the solvent on the reaction kinetics of DMACA (0.5%) with (+)-catechin (10 μg/ml) in the presence of 0.75 *M* H_2SO_4. The solvent was made up to 100% with water.

flavonoids with a carbonyl function at C4, *i.e.*, naringenin show a rather weak reaction (Table III) as already shown by Sarkar and Howarth[54]. The high sensitivity of indole was diminished by substitution at the pyrrole ring, *cf.*, tryptamine. Additionally, the relative sensitivity was affected by chromophoric groups and their binding sites.

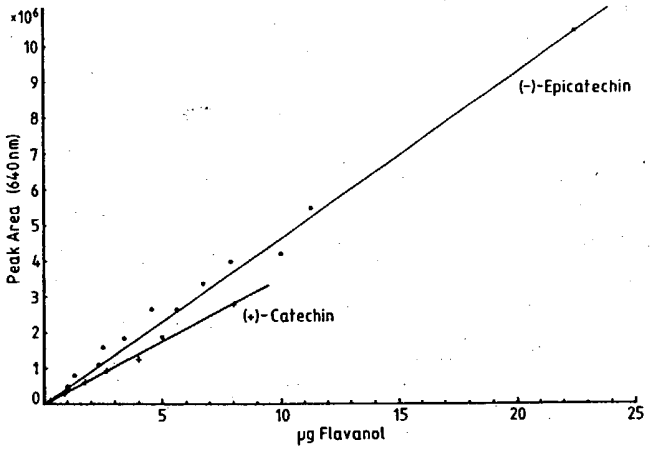

Fig. 3. Calibration graphs for (+)-catechin and (−)-epicatechin. Detection after chemical reaction with DMACA (1% in 1.5 *M* methanolic suphuric acid); for separation conditions see Experimental.

TABLE III

RELATIVE SENSITIVITY OF THE DMACA REAGENT TOWARDS VARIOUS SUBSTRATES

Flow injection conditions: solvent, 40% aqueous methanol; flow-rate 0.6 ml/min; reagent, 1% DMACA in 1.5 *M* sulphuric acid in methanol, flow-rate 0.6 ml/min.

Common name	*Structural name*	*Amount resulting in a peak of 0.04 a.u. at 640 nm (μM)*	*Relative sensitivity*[a]	*Absorbance maximum*	*Ratio 640/620 nm*
(−)-Epicatechin	3,3′,4′,5,7-Flavanpentol	$0.2 \cdot 10^{-3}$	1000 000	632	1.15
Indole		$0.4 \cdot 10^{-3}$	500 000	620	0.71
Orcinol	3,5-Dihydroxytoluol	$26 \cdot 10^{-3}$	7692	628	0.90
Phloroglucinol	1,3,5-Trihydroxybenzol	$35 \cdot 10^{-3}$	5714	618	0.51
Tryptamine	3-(2-Aminoethyl)indole	$38 \cdot 10^{-3}$	5263	576	0.72
Resorcinol	1,3-Dihydroxybenzol	$51 \cdot 10^{-3}$	3921	628	0.96
Pyrogallol	1,2,3-Trihydroxybenzol	$58 \cdot 10^{-3}$	3448	630	0.97
Catechol	1,2-Dihydroxybenzol	$60 \cdot 10^{-3}$	3333	617	0.42
Serotonine	5-Hydroxytryptamine	$160 \cdot 10^{-3}$	1250	594	0.67
Thymol[b]	2-Isopropyl-5-methylphenol	$800 \cdot 10^{-3}$	250	636	1.22
Naringenin	4′,5,7-Trihydroxyflavanone	1.8	111	570, 614	0.47
Naringin	Naringenin-7-glucosidorhamnoside	8.5	24	626	0.89
Terpinene[b]	1,3-*p*-Menthadiene	27	7.4	595	0.91
Citral[b]	3,7-Dimethyl-2,6-octadienal	54	3.7	575	0.76
Linalool	3,7-Dimethyl-1,6-octadien-3-ol	61	3.3	604	0.32
Camphene[b]	2,2-Dimethyl-3-methylenebicyclo[2.2.1]heptane	62	3.2	510	0.50
Geraniol[b]	*trans*-3,7-Dimethyl-2,6-octadienol	110	1.8	595	0.73
Guaiacol	*o*-Methylphenol	178	1.1	504	0.76
D-Limonene[b]	1,8(9)-*p*-Menthadiene	215	0.9	594	0.64
Menthol[b]	1-Methyl-4-isopropylcyclohexan-3-ol	384	0.5	600	0.53

[a] Calculated from the values in column 3 of the table as [epicatechin (μM)]/[other compound (μM)] · 10^6.

[b] Use of a butanol-containing solvent and reagent, to prevent demixing.

Another factor, which plays an important rôle in analyzing plant extracts, is the volatility of some compounds. During the concentration procedure with an evaporator, volatile substances such as indole and some terpenes disappear and do not disturb the catechin detection further. If any uncertainty about the catechin nature of a peak remains, an absorbance ratio between 640 and 620 nm for instance (Table III) may be helpful.

All these facts summarized in Table III led to the conclusion that the DMACA reagent described can be used for specific chemical reaction detection of catechin and oligomeric proanthocyanidins. It has been shown that the detector responded linearly to the signal (Fig. 3). For epicatechin the detection limit was 2.5 ng with a signal-to-noise ratio of 2.

Fig. 4 shows the separation and selective detection of the catechins and proanthocyanidins extracted from a chinese tea which is known to be rich in flavanols[66,67]. In Fig. 5 two chromatograms of the phenols of a bottled beer are compared with the detection at 280 nm (upper part) and 640 nm after chemical

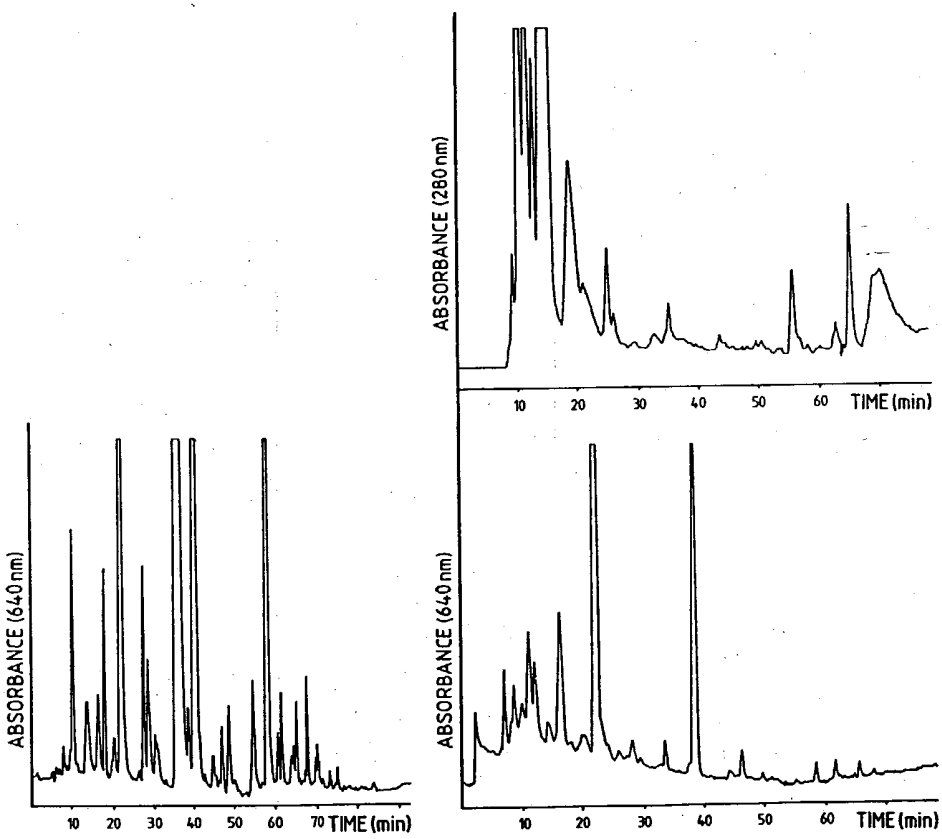

Fig. 4. HPLC separation and chemical reaction detection with DMACA of a phenolic extract from 1.5 mg dry chinese tea (*Camellia sinensis*).

Fig. 5. HPLC separation of the phenolic compounds of 0.5 ml bottled beer (concentrated to 10 μl) with detection at 280 nm (above) and after chemical reaction with DMACA at 640 nm (below).

reaction with DMACA. The UV-absorbance spectra of the main peaks of the 280-nm chromatogram (measured with a diode array detector) showed a maximum between 260 and 270 nm. From this one can conclude that the main peaks with short retention times are structurally not related to flavanols and that they overlap the catechins and proanthocyanidins. The latter were visualized with DMACA, resulting in the lower chromatogram (Fig. 5).

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REFERENCES

1 K. Freudenberg and K. Weinges, in T. A. Geissman (Editor), *The Chemistry of Phenolic Compounds*, Pergamon, Oxford, 1962, p. 197.
2 E. Haslam, *Chemistry of Vegetable Tannins*, Academic Press, London, 1966.
3 K. Weinges, *Phytochemistry*, 3 (1964) 263.
4 R. S. Thompson, D. Jacques, E. Haslam and R. J. N. Tanner, *J. Chem. Soc. Perkin Trans. I*, (1972) 1387.
5 L. Y. Foo and L. J. Porter, *Phytochemistry*, 19 (1980) 1747.
6 M. A. Joslyn and J. L. Goldstein, *Wallerstein Commun.*, 28 (1965) 143.
7 J. R. Ramirez-Martinez, A. Levi, H. Padua and A. Bakal, *J. Food Sci.*, 42 (1977) 1201.
8 A. G. H. Lea and G. M. Arnold, *J. Sci. Food Agric.*, 29 (1978) 478.
9 A. G. H. Lea, *J. Sci. Food Agric.*, 29 (1978) 471.
10 L. Jurd, *Am. J. Enol. Vitic.*, 20 (1969) 191.
11 A. G. H. Lea, P. Bridle, C. F. Timberlake and V. L. Singleton, *Am. J. Enol. Vitic.*, 30 (1979) 289.
12 M. Bourzeix, M. Clarens and N. Heredia, *Bull. Liaison Groupe Polyphenols*, 13 (1986) 403.
13 P. Mulkay and J. Jerumanis, *Cerevisia*, 8 (1983) 29.
14 J. A. Delcour and G. M. Tuytens, *J. Inst. Brew.*, 90 (1984) 153.
15 I. McMurrough, G. P. Hennigan and M. J. Loughrey, *J. Inst. Brew.*, 89 (1983) 15.
16 E. Haslam, T. H. Lilley and T. Ozawa, *Bull. Liaison Groupe Polyphenols*, 13 (1986) 352.
17 J. E. Beart, T. H. Lilley and E. Haslam, *Phytochemistry*, 24 (1985) 33.
18 G. Hill, F. Stellwaag-Kittler, G. Huth and E. Schlösser, *Phytopath. Z.*, 102 (1981) 102.
19 W. G. Berger and K. Herrmann, *Z. Lebensm.-Unters.-Forsch.*, 146 (1971) 266.
20 L. Narziss and H. Kessler, *Brauwissenschaft*, 24 (1971) 14.
21 A. G. H. Lea and C. F. Timberlake, *J. Sci. Food Agric.*, 29 (1978) 484.
22 J. Jerumanis, *Proc. Eur. Brew. Conv., Berlin*, (1979) 309.
23 F. Drawert, G. Leupold and V. Lessing, *Brauwissenschaft*, 30 (1977) 13.
24 W. Feucht, P. P. S. Schmid and E. Christ, *J. Plant Physiol.*, 125 (1986) 1.
25 W. Feucht, P. P. S. Schmid and E. Christ, *Obst Weinbau*, 22 (1985) 249.
26 M. E. Mace, A. A. Bell and R. D. Stipanovic, *Physiol. Plant Pathol.*, 13 (1978) 143.
27 T. Swain and W. E. Hillis, *J. Sci. Food Agric.*, 10 (1959) 63.
28 T. Swain and J. L. Goldstein, in J. B. Pridham (Editor), *Methods in Polyphenol Chemistry*, Pergamon, Oxford, 1964, p. 131.
29 J. A. Delcour and D. J. De Varebeke, *J. Inst. Brew.*, 91 (1985) 37.
30 H. Friedrich and H. Wiedemeyer, *Planta Med.*, 30 (1976) 223.
31 E. Haslam, *Phytochemistry*, 16 (1977) 1625.
32 H. A. Stafford and H. H. Lester, *Plant Physiol.*, 66 (1980) 1085.
33 A. S. L. Tirimanna and K. P. W. C. Perera, *J. Chromatogr.*, 58 (1971) 302.
34 H. A. Stafford and H. H. Lester, *Plant Physiol.*, 68 (1981) 1035.
35 A. W. Jaworski and C. Y. Lee, *J. Agric. Food Chem.*, 35 (1987) 257.
36 A. G. H. Lea, *J. Chromatogr.*, 238 (1982) 253.
37 I. McMurrough and J. McDowell, *Anal. Biochem.*, 91 (1978) 92.

38 I. McMurrough, *Proc. Eur. Brew. Conv., Berlin*, (1979) 321.
39 H. Engelhardt and R. Klinkner, *Fresenius' Z. Anal. Chem.*, 319 (1984) 277.
40 C. Hartwich and M. Winckel, *Arch. Pharm. (Weinheim Ger.)*, (1904) 462.
41 O. Lindt, *Z. Wiss. Mikroskopie Technik*, 2 (1885) 495.
42 T. von Fellenberg, *Chem.-Zt.*, 88 (1910) 791.
43 T. von Fellenberg, *Mitt. Lebensm. Hyg.*, 1 (1910) 311.
44 M. M. Raciborski, *Anz. Akad. Wiss. Krakow*, (1906) 553.
45 M. Joachimowitz, *Biochem. Z.*, 82 (1917) 324.
46 J. Kolsek and M. Perpar, *Fresenius' Z. Anal. Chem.*, 167 (1958) 161.
47 L. Rosenthaler, *Fresenius' Z. Anal. Chem.*, 44 (1905) 292.
48 L. Ekkert, *Pharm. Zentralhalle*, 68 (1927) 577.
49 L. Ekkert, *Pharm. Zentralhalle*, 69 (1928) 289.
50 T. Swain and W. E. Hillis, *J. Sci. Food Agric.*, 10 (1959) 63.
51 M. Thies and R. Fischer, *Mikrochim. Acta*, (1971) 9.
52 M. E. Mace and C. R. Howell, *Can. J. Bot.*, 52 (1974) 2423.
53 R. O. Gardner, *Stain Technol.*, 50 (1975) 315.
54 S. K. Sarkar and R. E. Howarth, *J. Agric. Food Chem.*, 24 (1976) 317.
55 W. Feucht and P. P. S. Schmid, *Gartenbauwiss.*, 48 (1983) 119.
56 M. L. Price, S. Van Scoyoc and L. G. Butler, *J. Agric. Food Chem.*, 26 (1978) 1214.
57 J. A. Delcour, in H. F. Linskens and J. F. Jackson (Editors), *Beer Analysis*, Springer, Berlin, 1988, p. 225.
58 F. C. Happold and L. Hoyle, *Biochem. J.*, 28 (1934) 1171.
59 J. Harley-Mason and A. A. P. G. Archer, *Biochem. J.*, 69 (1958) 60.
60 J. M. Turner, *Biochem. J.*, 78 (1961) 790.
61 H. Engelhardt and U. D. Neue, *Chromatographia*, 15 (1982) 403.
62 S. R. Finn and J. W. James, *J. Appl. Chem.*, 6 (1956) 466.
63 W. E. Hillis and G. Urbach, *J. Appl. Chem.*, 9 (1959) 474.
64 P. Ribereau-Gayon, *Plant Phenolics*, Oliver & Boyd, Edinburg, 1972.
65 H. Geiger, in C. F. Van Sumere and P. J. Lea (Editors), *The Biochemistry of Plant Phenolics*, Clarendon, Oxford, 1985, p. 45.
66 A. G. Brown, W. B. Eyton, A. Holmes and W. D. Ollis, *Phytochemistry*, 8 (1969) 2333.
67 A. C. Hoefler and P. Coggon, *J. Chromatogr.*, 129 (1976) 460.

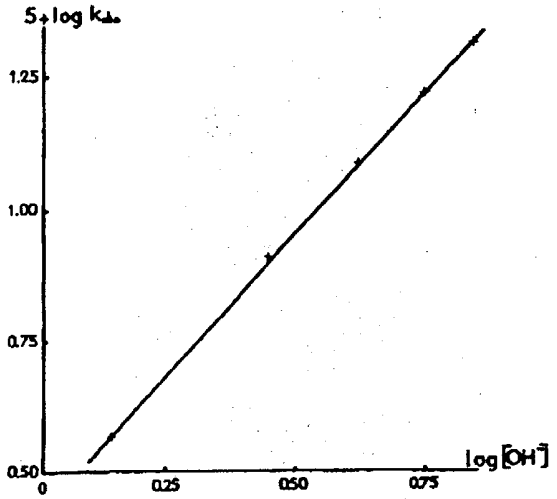

Figure 3. Plot of the logarithms of the observed rate constants vs. the logarithms of hydroxide ion concentration for the hydrolysis of STB to BBU at 25° C in water-methanol.

$[OH^-]$ is essentially 1.0 (Figure 3), indicating that the reaction has a simple first-order dependence on hydroxide ion concentration. The opening of the triazine ring of STB results in BBU formation in the reaction medium. This interpretation is consistent with the observations of White et al. (1973). The nucleophilic attack of hydroxide ion at the carbonyl next to the nitrogen atom N_1 of the benzimidazole ring causes the opening of the triazine ring of STB. This reaction is much slower than the cyclization of benomyl to STB: when $[OH^-] = 1.0$, $k_{obsd} = 0.2 \times 10^{-4}$ s^{-1}; $[OH^-] = 7.0$, $k_{obsd} = 2 \times 10^{-4}$ s^{-1}.

CONCLUSIONS

These results are of theoretical as well as of practical value and allow a better understanding of the chemical fate of benomyl in various conditions.

Benomyl can, in some circumstances, be submitted to alkaline conditions. Some anticryptogamic agents, such as the Bordeaux mixture, are alkaline. Local alkaline conditions can prevail in soils after recent or excessive liming. Heat treatments and alkaline peeling solutions are often used in fruit processing. In these various cases, the involvement of the reaction pathways leading to STB and BBU is quite possible. The environmental impact of either STB or BBU is not well known to date. However, it seems that, in normal practical use situations, the residue levels of benomyl or its derivatives are very low. As for the aerial parts of plants, even after alkaline treatments, intact benomyl constitutes the major component of the local residue on the leaves (Baude et al., 1973); the fast drying-out of the spray droplets prevents noticeable further degradation of benomyl.

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LITERATURE CITED

Banthorpe, D. V., "Elimination Mechanisms", Elsevier, London, 1963, p 5.
Baude, F. J., Gardiner, J. A., Han, J. C. Y., *J. Agric. Food Chem.* 21, 1084 (1973).
Bose, E. A., White, E. R., U.S. Patent 3725406, April 3, 1973.
Christenson, I., *Acta Chem. Scand.* 18, 904 (1964).
Keith, L. H., Alford, A. L., *J. Assoc. Off. Anal. Chem.* 53, 157 (1970).
Tobias, P. S., Kezdy, F. J., *J. Am. Chem. Soc.* 91, 5171 (1969).
White, E. R., Bose, E. A., Ogawa, J. M., Manji, B. T., Kilgore, W. W., *J. Agric. Food Chem.* 21, 616 (1973).

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Specificity of the Vanillin Test for Flavanols

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The reaction with vanillin in acidic solution was previously considered to be a specific test for flavanols. In this work the specificity of the vanillin reaction was reexamined by testing the reactivity of 15 flavonoid and two chromone compounds. In addition to flavanols, the dihydrochalcones phloretin and phloridzin gave significant color development. Flavanone and flavanonol aglycones reacted weakly. The structural requirements for a positive reaction have been deduced. The vanillin–HCl screening test, used by plant breeders, has been modified to prevent the possible occurrence of a false positive interpretation due to the presence of anthocyanins in plant materials. The anthocyanidins cyanidin, pelargonidin, and peonidin were identified in alfalfa (*Medicago sativa* L.) herbage, and several other herbaceous legumes were examined for the presence of anthocyanins. The possibility of interference by dihydrochalcones or anthocyanins should be considered when the vanillin–HCl reaction is used for the detection and quantitative analysis of flavanols in plant materials.

Alfalfa (*Medicago sativa* L.), red clover (*Trifolium pratense* L.), and white clover (*T. repens* L.) are legume forages which may cause ruminant bloat when they are grazed by cattle or sheep, but sainfoin (*Onybrychis viciaefolia* Scop.) and birdsfoot trefoil (*Lotus corniculatus* L.) are legume forages which do not cause bloat. The observation that protein precipitants are present in sainfoin and birdsfoot trefoil herbage, but absent from alfalfa, red clover, and white clover herbage, has led to the conclusion that protein precipitants are responsible for the nonbloating property of sainfoin and trefoil (Gutek et al., 1974; Jones and Lyttleton, 1971). Hence it would be of great benefit if plant breeders could introduce protein precipitants into alfalfa, red clover, and white clover to

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prevent the occurrence of bloat in ruminant animals grazing these species.

The ability to precipitate proteins is a property of tannins. Unfortunately it is difficult to define the term "tannin" and this has been a cause of confusion in the scientific literature. In this report we use the term according to the definition given by Swain and Bate-Smith (1962), i.e., water-soluble, phenolic compounds, with molecular weights between 500 and 3000, and having the ability to precipitate proteins. The tannins of herbaceous legumes are flavolans which are polymers of flavan-3-ols and flavan-3,4-diols (Bate-Smith, 1973; Jones et al., 1973).

In a survey of legume forages Jones et al. (1973) detected tannins by extraction of proteins from herbage in the presence and absence of polyvinylpyrrolidone (PVP). The presence of tannins was indicated by extraction of a larger amount of protein in the presence of PVP compared to extraction in the absence of PVP. In the same study they obtained a positive vanillin–HCl test for flavanols in every species which contained tannins as judged by the PVP test. Similar results have been obtained in this laboratory (Howarth and Goplen, 1974). Jones et al. (1973) obtained a negative result when alfalfa was examined by the vanillin–HCl test. However, Milić (1972) and Delić (1972) used the vanillin–HCl reaction and reported the presence of flavanols in alfalfa. Lehman (1974) has selected alfalfa strains on the basis of response to the vanillin–HCl test. These conflicting results led us to examine the specificity of the vanillin–HCl test, particularly concerning its application to crude leaf extracts of forage legumes for plant breeding purposes.

The vanillin reagent reacts in an approximately stoichiometric manner with compounds containing meta-oriented di- or trihydroxy substituents on the benzene rings (Swain and Goldstein, 1963). A positive reaction is indicated by the appearance of a light pink to deep cherry red coloration. In the case of flavonoids, 5,7-dihydroxy compounds are deactivated by the presence of a carbonyl group in the C-4 position (Ribereau-Gayon, 1972). Therefore, the reaction has been applied as a specific test for flavanols (Swain and Hillis, 1959) because they lack the carbonyl group at the C-4 position. The reaction has been adapted to a screening test for plant breeding purposes by crushing leaves between two layers of filter or chromatography paper and then applying the reagent to the imprint on the paper (Burns, 1963; Jones et al., 1973).

EXPERIMENTAL SECTION

The specificity of the vanillin reaction was investigated by testing a number of flavonoid compounds which varied in the oxidation level of the middle heterocyclic ring. Several chromone compounds which lack the B ring of the flavonoid nucleus were also tested. The test compounds were obtained from K and K Rare and Fine Chemicals, Pfaltz and Bauer Inc., and Fluka AG.

The test compounds were dissolved in distilled water at concentrations of 8 to 33 μg/mL. The vanillin reagent contained 1% vanillin in 70% (v/v) sulfuric acid (Swain and Hillis, 1959). Four milliliters of vanillin reagent was mixed with 3.0 ml of sample solution. Absorbance readings were taken at 500 nm with the spectrophotometer zeroed against a reagent blank.

Plant material was tested for flavolans by crushing leaves or stems between two layers of 3MM chromatography paper and applying vanillin solution to the imprint on one of the layers (Jones et al., 1973). The vanillin solution contained 2 vol of 10% w/v vanillin in ethanol mixed with 1 vol of concentrated HCl. In this work it was necessary to apply a control solution (2 vol of ethanol in 1 vol of concentrated HCl) to the imprint on the second paper layer to avoid the possibility of obtaining a false positive response.

Anthocyanins were extracted from 10 g of herbage by homogenization with 30 ml of methanol–water (1:1). The homogenizer (Sorvall) was cooled in ice and operated for 2 min. Homogenates were filtered through glass-fiber disks; the filtrates were concentrated to about 2 ml in a rotary evaporator, and centrifuged for 3 min at 600g. The supernatant solutions were streaked on Whatman 3MM chromatography paper and the chromatograms were developed in butanol–acetic acid–water (4:1:5, upper layer). The anthocyanins appeared as colored bands on the chromatograms. They were identified by comparison with R_f values reported in the literature and by their absorption spectra.

The anthocyanins from alfalfa herbage were eluted from the chromatograms with 1% HCl in methanol (v/v) and an aliquot was rechromatographed on a cellulose thin-layer chromatogram developed in acetic acid–HCl–H_2O (15:3:82). Another aliquot was heated to 100 °C for 30 min in 3 N HCl to hydrolyze the anthocyanins. The anthocyanidins so obtained were extracted into amyl alcohol, and their R_f values were determined on a cellulose thin-layer chromatogram developed in acetic acid–HCl–water (30:3:10). Another aliquot of the amyl alcohol extract was spotted on Whatman 3MM chromatography paper and developed in 1% HCl. The anthocyanidin bands were eluted into 1% HCl in methanol for measurement of their visible absorption spectra. Standard anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, and peonidin) were chromatographed along with the anthocyanidins from alfalfa.

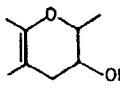
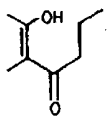
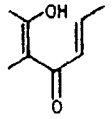
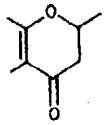
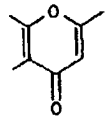
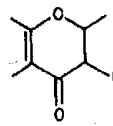
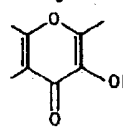
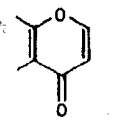
RESULTS AND DISCUSSION

We considered the possibility that the oxidation level of the middle heterocyclic ring may affect the reactivity of flavonoids with the vanillin reagent. The reactivities of flavonoid glycosides and of chromones, which lack the flavonoid B ring, were also tested. Seventeen compounds representing flavanols, flavanones, flavones, flavanonols, flavonols, dihydrochalcones, chalcones, and chromones were examined. The chemical structures of these compounds are shown in Figure 1 and Table I, and their reactions with the vanillin reagent are given in Table I. Six compounds gave positive reactions but there were large differences in the intensities of color development. Molar extinction coefficients of the reactive compounds are shown in Table II.

Our results show that the vanillin reagent is not completely specific for flavanols. Catechin, a flavanol, gave the greatest intensity of color development, but phloretin, a dihydrochalcone, gave a color intensity in the same order of magnitude as catechin. When phloretin concentration in the sample solution was 16 μg/ml a red complex precipitated after addition of the vanillin reagent. There was no visible precipitate at a phoretin concentration of 8 μg/ml. Phloridzin, a dihydrochalcone glycoside, gave an intermediate color intensity while naringenin, hesperetin, and dihydroquercetin gave small amounts of color.

Swain and coworkers (Swain and Hillis, 1959; Goldstein and Swain, 1963) tested the specificity of the vanillin reaction using a variety of compounds. Our results confirm their finding that chalcones, flavonols, and flavanone 7-glycosides do not give a color reaction (Swain and Hillis, 1959). However, we have tested a greater variety of flavonoid compounds and our observations provide new information on the structural requirements for a positive

Table I. Responses of Some Flavonoid and Chromone Compounds to the Vanillin Test for Flavanols

Class	Structure of C_3 unit	Compound	Reaction with vanillin
Flavanols		Catechin (5,7,3',4'-tetrahydroxyflavan-3-ol)	+++
Dihydrochalcones		Phloretin (4,2',4',6'-tetrahydroxydihydrochalcone)	+++
		Phloridzin (phloretin 2'-glucoside)	++
Chalcone		Butein (3,4,2',4'-tetrahydroxychalcone)	–
Flavanones		Naringenin (5,7,4'-trihydroxyflavanone)	+
		Naringin (naringenin 7-rutinoside)	–
		Hesperetin (5,7,3'-trihydroxy-4'-methoxyflavanone)	+
		Hesperidin (hesperetin 7-rutinoside)	–
Flavones		7-Hydroxyflavone	–
		5,7-Dihydroxyflavone	–
Flavanonol		Dihydroquercetin	+
Flavonols		Kaempferol (5,7,4'-trihydroxyflavonol)	–
		Quercetin (5,7,3',4'-tetrahydroxyflavonol)	–
		Quercitrin (quercetin 3-rhamnoside)	–
		Rutin (quercetin 3-rutinoside)	–
Chromones		Eugenin (2-methyl-5-hydroxy-7-methoxychromone)	–
		2-Methyl-5,7-dihydroxychromone	–

reaction by flavonoid compounds.

Vanillin is protonated in acid solution, giving a weak electrophilic radical which reacts with the flavonoid ring at the 6 or 8 position. This intermediate compound is dehydrated to give a red colored compound (Ribereau-Gayon, 1972). Table I shows that a single bond between

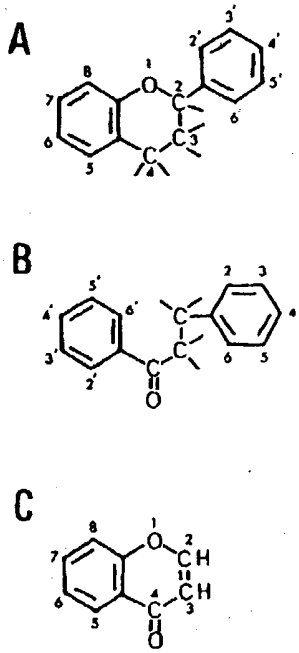


Figure 1. Numbering systems for: (A) most flavonoids; (B) chalcones, dihydrochalcones; (C) chromones.

Table II. Molar Extinction Coefficients of the Colored Products Formed from Flavonoid Compounds with the Vanillin Reagent

Compound	$\epsilon \times 10^{-3}$
Catechin	33.8
Phloretin	25.3
Phloridzin	9.1
Naringenin	2.5
Hesperetin	2.8
Dihydroquercetin	2.5

C-2 and C-3 is an essential requirement for a positive reaction. Chalcones, flavones, flavonols, and chromones are completely inactivated by a double bond in this location. Inactivation is probably due to decreased electron density in the A ring because of electron delocalization. Only small amounts of color were given by naringenin, hesperetin, and dihydroquercetin indicating nearly complete inactivation by the electron-withdrawing effect of the carbonyl group at C-4. However, much less inactivation by the carbonyl occurred with the dihydrochalcones which were activated by an additional free hydroxy group at C-6'. The flavanone glycosides, naringin and hesperidin, did not react, indicating the requirement for a free hydroxy group at C-7. The glycoside phloridzin produced less color than the corresponding aglycone, phoretin, probably because glycosylation decreased electron density at C-2' or because the glucosyl group sterically hindered substitution at C-3'. In summary, the essential structural requirements for reaction of the flavonoids with the vanillin reagent are a single bond at the 2,3 position and free meta-oriented hydroxy groups on the B ring. Substantial amounts of color development are therefore given by the flavanols and dihydrochalcones.

These results raise the possibility of interference by dihydrochalcones when the vanillin–HCl test is used for the detection or quantitative determination of flavanols in plant materials. Although considerable quantities of phloridzin are present in apple and pear leaves (Williams, 1966), the dihydrochalcones are less widely distributed than flavanols (Harborne and Simmonds, 1964). Accordingly, a positive vanillin–HCl reaction is probably

Table III. Anthocyanins from Alfalfa Herbage

Paper chromatography		Thin-layer chromatography,	Identity of
Band no.	R_f value[a]	R_f[b]	anthocyanidins[c]
1	0.03	0.90	Cy
2	0.07	0.66 and 0.90	Cy
3	0.17	0.66 and 0.90	Cy, Pg, Pn
4	0.24	0.74 and 0.90	Cy, Pg, Pn
5	0.37	0.08	Cy

[a] Solvent: butanol–acetic acid–water (4:1:5), upper layer. [b] Solvent: acetic acid–HCl–water (15:3:82). [c] Abbreviations: cyanidin (Cy), pelargonidin (Pg), and peonidin (Pn).

attributable to flavanols but additional tests by paper chromatography would be necessary to confirm their presence and to establish that dihydrochalcones are not present.

When we used the vanillin–HCl screening test (Jones et al., 1973) to survey a number of alfalfa cultivars and strains, several plants gave a pink color which appeared to be a positive reaction. However, further examination of these plants by testing for protein precipitants (Jones and Lyttleton, 1971) or by paper chromatography of methanol extracts (Sarkar et al., 1976) failed to confirm the presence of flavanols or tannins. Since anthocyanins give a pink to red color in acidic solutions, alfalfa herbage was examined for the presence of anthocyanins. Paper chromatography of extracts from alfalfa herbage showed the presence of five colored bands (Table III), three of which gave two bands by thin-layer chromatography. When the colored bands were eluted from paper chromatograms and hydrolyzed in HCl, three anthocyanidins were obtained: cyanidin, peonidin, and pelaragonidin. Delphinidin, petunidin, and malvidin occur in alfalfa petals (Cooper and Elliott, 1964) but to the best of our knowledge this is the first report on identification of the anthocyanidins in alfalfa herbage. Several other herbaceous legumes were examined for the presence of anthocyanins. Sericea (*Lespedeza cuneata* Don.), birdsfoot trefoil (*Lotus corniculatus* L.), small hop clover (*Trifolium dubium* Sibth.), and large hop clover (*T. campestre* Schreb.) contained an anthocyanin which was identified as cyanidin 3,5-diglucoside. Identification was on the basis of R_f value and absorption spectrum. Anthocyanins were not detected in rabbit foot clover (*T. arvense* L.) and crownvetch (*Coronilla varia* L.).

We introduced a modification to the vanillin–HCl screening test to avoid a false, positive interpretation due to the presence of anthocyanins in the plant materials. A control solution containing HCl in ethanol was applied to the leaf imprint on the second layer of chromatography paper. If a red coloration results from the presence of anthocyanins, it will appear on the leaf imprint treated with this control solution, as well as on the imprint treated with the complete reagent solution. Red color resulting from the presence of flavolans will appear on the imprint treated with the complete reagent solution but not on the imprint treated with the control solution. The modified vanillin–HCl screening test has been used to survey many alfalfa plants and we have not detected the presence of flavolans (Howarth and Goplen, 1974). Thus in this work, as well as that reported elsewhere (Sarkar et al., 1976), we have been unable to confirm the occurrence of flavanols in alfalfa as reported by Milić (1972) and Delić (1972). Possibly their extracts contained anthocyanins.

In conclusion, the vanillin–HCl reaction is useful for the detection and quantitative analysis of flavanols in plants but the possibility of interference by dihydrochalcones and anthocyanins should be considered.

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LITERATURE CITED

Bate-Smith, E. C., *Phytochemistry* 12, 1809 (1973).
Burns, R. E., *Ga. Agric. Exp. Stn. Tech. Bull.* No. 32 (1963).
Cooper, R. L., Elliott, F. C., *Crop Sci.* 4, 367 (1964).
Delić, I. L., Ph.D. Thesis, Novi Sad, Yugoslavia, 1972.
Goldstein, J. L., Swain, T., *Phytochemistry* 2, 371 (1963).
Gutek, L. H., Goplen, B. P., Howarth, R. E., McArthur, J. M., *Crop Sci.* 14, 495 (1974).
Harborne, J. B., Simmonds, N. W., "Biochemistry of Phenolic Compounds", Harborne, J. B., Ed., Academic Press, New York, N.Y., 1964, pp 77–127.
Howarth, R. E., Goplen, B. P., Agriculture Canada Research Station, Saskatoon, Canada, unpublished results, 1974.
Jones, W. T., Anderson, L. B., Ross, M. D., *N. Z. J. Agric. Res.* 16, 441 (1973).
Jones, W. T., Lyttleton, J. W., *N. Z. J. Agric. Res.* 14, 101 (1971).
Lehman, W. F., University of California, El Centro, personal communication, 1974.
Milić, B. L., *J. Sci. Food Agric.* 23, 1151 (1972).
Ribereau-Gayon, P., "Plant Phenolics", Oliver and Boyd, Edinburgh, 1972.
Sarkar, S. K., Howarth, R. E., Goplen, B. P., manuscript submitted for publication to *Crop Sci.*, 1976.
Swain, T., Bate-Smith, E. C., *Comp. Biochem.* 3, 755–809 (1962).
Swain, T., Goldstein, J. L., "Methods in Polyphenol Chemistry", Pridham, J. B., Ed., MacMillan, New York, N.Y., 1963, pp 131–146.
Swain, T., Hillis, W. E., *J. Sci. Food Agric.* 10, 63 (1959).
Williams, A. H., "Comparative Phytochemistry", Swain, T., Ed., Academic Press, New York, N.Y., 1966, pp 297–307.

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Review

Analysis of condensed tannins: a review

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Abstract

Plant condensed tannins (proanthocyanidins, PAs) have both positive and negative effects on feed digestibility and animal performance, depending both on the quantity and biological activity of the tannins that are present. In this review, the chemistry and analysis of condensed tannins (PAs) are examined. Our first focus is on the complexity of the structures of condensed tannins and our second emphasis is on the analytical methods used to evaluate tannins. The section on methods is subdivided into a discussion of methods to determine the amount of condensed tannins or total phenolics in a sample and a section on methods to measure biological activity. The methods to measure reactivity include assays involving protein binding and precipitation, as well as those that involve enzymatic and microbial inhibition. The last section of the paper discusses structure–activity relationships and provides information on how to select appropriate assays for measurement of the quantity and activity of condensed tannins.

Keywords: Tannin; Assay; Structure; Enzyme inhibition; HPLC

1. Introduction

Condensed tannins (or proanthocyanidins, PAs) comprise a group of polyhydroxyflavan-3-ol oligomers and polymers linked by carbon–carbon bonds between flavanol subunits (Figs. 1 and 2). The reactivity of PAs with molecules of biological significance has important nutritional and physiological consequences. Their multiple phenolic hydroxyl groups lead to the formation of complexes with proteins (Hagerman et al., 1998; Harborne, 1998; Naczk et al., 1996), with metal ions (Foo et al., 1997; Scalbert, 1991; Van Acker et al., 1998) and with other macromolecules like polysaccharides (Mueller-Harvey and McAllan, 1992).

Analysis of condensed tannins is complicated by the diversity of structures found within this group of compounds. To clarify the discussion of methods, we therefore

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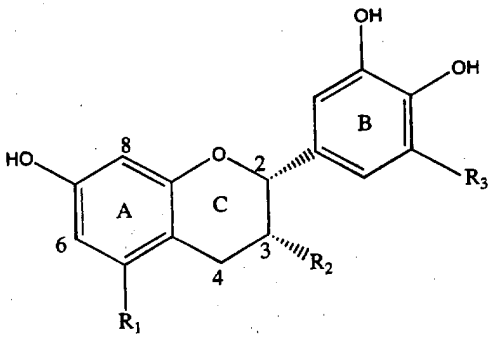
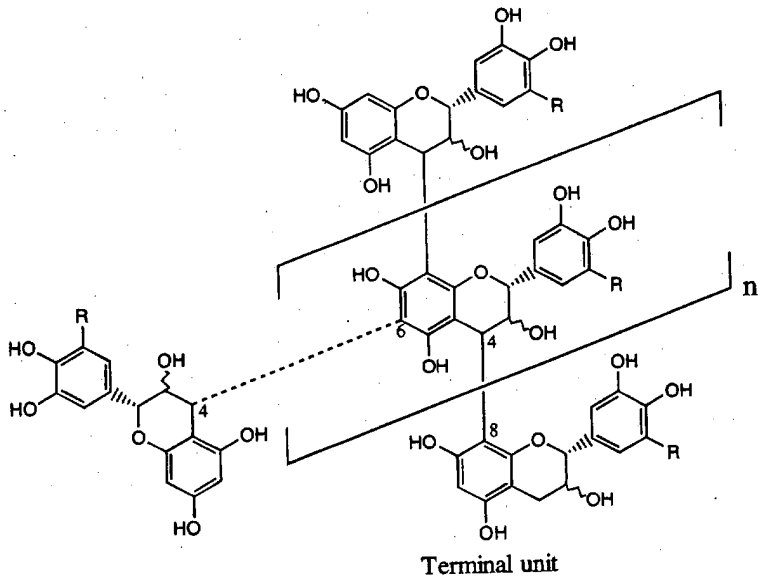


R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Fig. 1. The basic repeating unit in condensed tannins. If $R_1 = R_2 = OH$, $R_3 = H$, then the structure is that for (-)-epicatechin. The groups at R_1 and R_3 for other compounds are indicated below the structure. R_2 = O-galloyl in the catechin gallates.



Terminal unit

Fig. 2. Model structure for a condensed tannin. If R = H or OH then the structure represents a procyanidin or prodelphinidin. The 4 → 6 linkage (dotted line) is an alternative interflavan bond. The terminal unit is at the bottom of such a multi-unit structure.

review briefly the topic of condensed tannin structure and refer the reader to other sources (Harborne and Baxter, 1999; Haslam, 1989; Waterman and Mole, 1994) for more detailed discussions.

This review is divided into three main sections. Section 2 describes tannin structures, Section 3 covers analytical methods, and Section 4 contains speculations on structure–activity relationships and their bearing on the choice of tannin assays.

2. Structures of condensed tannins

Condensed tannins are polymers of flavanol units. The catechin monomer has asymmetric centers at positions 2 and 3 in ring C (Fig. 1). Although variations in the stereochemistry at these positions do occur in natural tannins, observations on model compounds suggest that these variations have relatively little effect on most of the reactions used for tannin assays (Schofield, unpublished data). The interested reader may consult Haslam (1989) for more information on structures. The substituent groups R_1, R_2, R_3 in Fig. 1 may have a significant effect on tannin reactivity. R_2 is an OH group, sometimes esterified to gallic acid (e.g. as in epigallocatechin gallate ($R_1 = R_3 = OH$, R_2 = O-galloyl), a major polyphenolic ingredient of green tea). The presence of gallate esters in tannins may change their biological properties significantly (Hagerman, 1992). R_1 and R_3 may be different combinations of OH and H groups that lead to different classes of flavanol polymers (Fig. 1) with differing responses to various assay reagents. Thus procyanidins can be distinguished from prodelphinidins because prodelphinidins have a hydroxyl group at position 5 of the B ring. For example, an increase in the prodelphinidin/procyanidin ratio in a condensed tannin increases the ability of that tannin to complex with proteins (Aerts et al., 1999).

Oxidative coupling between flavanol monomers occurs most commonly between positions 4 and 8, but may also involve positions 4 and 6 of the monomer (Fig. 2) and other positions too. Whereas the total number of monomeric flavanol units in extractable grape seed tannins ranged from 2 to 17 (Prieur et al., 1994), much higher degrees of polymerization may exist, but these polymers tend to be insoluble and difficult to study.

3. Analytical methods

The study of condensed tannins has been difficult because of the structural complexity of these compounds. Many analytical methods have been used to quantify tannins in plant materials. Commonly used methods include oxidative depolymerization of PA, reactions of the A ring (Fig. 1) with an aromatic aldehyde, and oxidation–reduction reactions (Waterman and Mole, 1994). Other methods involve acid cleavage reactions, precipitation reactions, enzyme and microbial inhibition and gravimetric procedures. The objective of this paper is to discuss some of these analytical methods highlighting the problems associated with them and their applicability. The chemical reactions underlying each kind of assay are presented. We note that much work still needs to be done to

Table 1
Summary of condensed tannin analyses on certain tropical legumes

Legume	Condensed tannins (g kg^{-1} DM)[a]			Total[b]
	Soluble	Insoluble	NDF-bound	
D. ovalifolium (12)[c]	63–272	12–35	1.2–9.7	76–299
Gliricidia sepium (9)	0–44	21–121	2–29	21–121
Manihot esculenta (4)	22–126	12–22	0.3–1.2	35–148

[a] Butanol–HCl assays using internal standards isolated with ytterbium. Summarized from Giner-Chavez et al. (1997).
[b] Total tannin content, soluble + insoluble (does not include NDF-bound).
[c] Number of different samples analyzed.

improve the existing methods and to develop new ones for effective understanding of tannin biology and biochemistry.

The initial harvesting, drying and extraction methods have significant effects on condensed tannin analyses. These topics have been comprehensively reviewed by Waterman and Mole (1994) and Mueller-Harvey (2001). Most condensed tannin assays have been done on the soluble material extracted with aqueous acetone or methanol. One may also wish to evaluate tannins insoluble in these solvents. This fraction may include higher molecular weight tannins and tannins bound to fiber. Methods for the analysis of insoluble tannins include the use of ^{13}C-NMR (Makkar et al., 1999) and application of the butanol–HCl reaction to insoluble plant materials (Reed et al., 1982; Giner-Chavez et al., 1997).

Before embarking on a detailed survey of analytical methods, we should note the considerable influence of forage provenance on condensed tannin content. In Table 1, we summarize data from Giner-Chavez et al. (1997). It is clear from these data that, even within a species, the condensed tannin content can vary over at least a 4- to 6-fold range depending on plant provenance.

3.1. Acid–butanol assay

This colorimetric reaction has a long history (Waterman and Mole, 1994) and uses an acid-catalyzed oxidative depolymerization of condensed tannins to yield red anthocyanidins. It is diagnostic for the polyflavan structure. The reaction is summarized in Fig. 3 (Haslam, 1989). Porter et al. (1986) described a procedure to use the reaction for quantitative analysis of condensed tannins. Users of this procedure should be aware of the following limitations and considerations, summarized, in part, from Waterman and Mole (1994):

1. The amount of water in the reaction medium is reported to be critical in color formation and therefore in the quantitative determination of PAs (Waterman and Mole, 1994; Hagerman and Butler, 1994; Dalzell and Kerven, 1998). Increasing the water content from 2 to 6% (v/v) in a methanol-based reaction medium suppressed the anthocyanidin yield of condensed tannins from *Leucaena* spp. (Dalzell and Kerven, 1998). The traditionally recommended water content is 6% (Porter et al., 1986; Waterman and Mole, 1994). However, Hagerman and Butler (1994) noted that even when there is a substantial amount of water in the medium, the assay is sensitive enough to give satisfactory results provided that the standards contain similar amounts of water. The amount of water present may lead to variable color development when different plant species are used (Dalzell and Kerven, 1998).
2. The ease with which the interflavan bond is cleaved by acid varies widely; 4 → 6 bonds (Fig. 2) are more resistant to this cleavage than are the more usual 4 → 8 bonds.

   The nature of the A ring substituent (Fig. 1) also affects acid lability. For example, in quebracho tannin, which is frequently used as a standard in this condensed tannin assay, R_1 is H rather than OH. This change increases the acid stability of the interflavan bond and reduces the color yield from this tannin (Giner-Chavez et al., 1997).
3. The number of phenolic groups in rings A and B (Fig. 1) affects the wavelength of the absorbance maximum and the extinction coefficients of the anthocyanidin products. For example, cyanidin and delphinidin (2 vs 3 phenolic OH in B) have λ_{max} at 545 and 557 nm, respectively (Hemingway, 1989).
4. Color yield is not always linear with the amount of tannin input. Larger amounts may give a lower unit color yield (Waterman and Mole, 1994).
5. The presence of transition metal ions in the assay medium is an important factor in color development (Dalzell and Kerven, 1998; Hagerman et al., 1997; Scalbert, 1992). Porter et al. (1986) reported that Fe^{3+} was the most efficient transition metal ion in catalyzing color formation in the butanol–HCl reaction. At least one recent report has clouded this picture. Dalzell and Kerven (1998) found the use of Fe^{3+} at concentrations greater than 15 μg/ml decreased color development from *Leucaena* spp. condensed tannins. They speculated that excess Fe^{3+} either inhibited tannin depolymerization or reduced the absorbance of the anthocyanidin products. Inconsistent color development has led some investigators to discontinue the use of metal ion catalysts (Terrill et al., 1992). Additional research is needed to clarify the role of metal ions in anthocyanidin production.

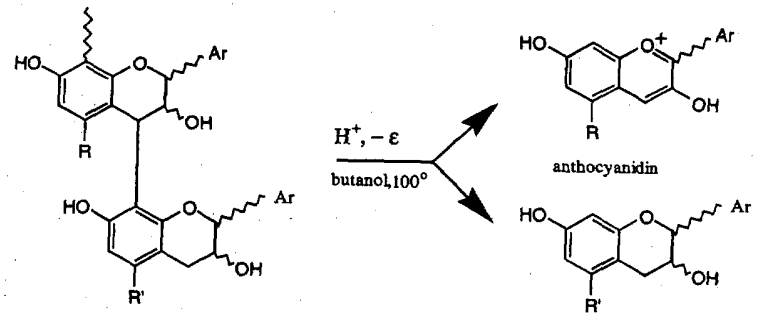


Fig. 3. Chemistry of the acid–butanol reaction. Note that the reaction involves oxidation and that the terminal unit does not give a colored anthocyanidin product structure.

6. Another important factor is the ratio of acid–butanol to sample medium in the reaction mixture (Waterman and Mole, 1994). A ratio of acid–butanol to sample medium of 6:1 has been proposed (Hagerman and Butler, 1994; Hagerman, 1998). When the ratio of reagent to sample was increased from 4:1 to 6:1, the color yield decreased (Dalzell and Kerven, 1998). Other important considerations in the acid–butanol assay are strict regulation of the temperature and length of the reaction time (Scalbert, 1992). The presence of ascorbic acid, an anti-oxidant, in the reaction medium increased color development (Dalzell and Kerven, 1998). This observation is unexpected because earlier research showed that oxygen is needed for anthocyanidin production (Porter et al., 1986).
7. The choice of standards remains an unresolved issue due to the heterogeneity of condensed tannins and the lack of appropriate standards for their quantification. Cyanidin, delphinidin and quebracho tannin have been used as standards (Hagerman and Butler, 1989; Scalbert, 1992; Giner-Chavez et al., 1997). While cyanidin would be an appropriate standard for cyanidin-yielding PAs, it is less suitable for prodelphinidins (Giner-Chavez et al., 1997; Scalbert, 1992). Condensed tannins may be underestimated if cyanidin is used as a standard because the yield of cyanidin from some tannins may not be quantitative (Scalbert, 1992). To minimize the problems from use of inappropriate standards, the use of internal standards derived from the plant materials under study has been proposed (van Hoven and Furstenburg, 1992; Waterman and Mole, 1994; Giner-Chavez et al., 1997; Hagerman, 1998). Fig. 4 shows the relationship between quantity of tannins and absorbance of tannins from three

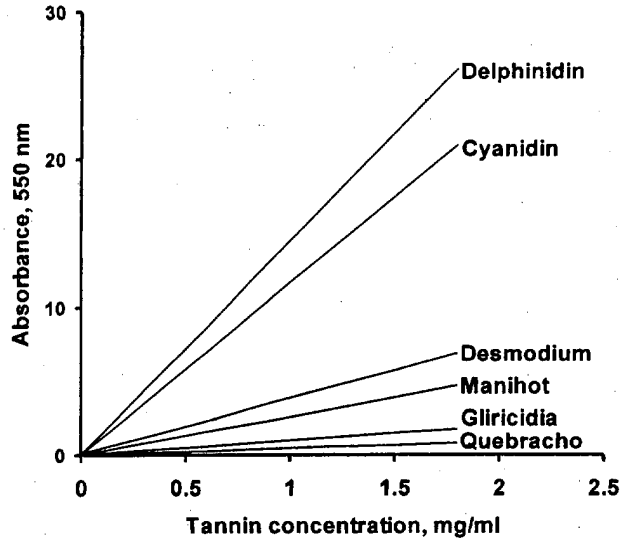


Fig. 4. Relationship between tannin concentration and absorbance at 550 nm (A_{550}) in the acid–butanol assay. Desmodium: *D. ovalifolium*; Manihot: *M. esculenta*; Gliricidia: *G. sepium* (Giner-Chavez et al., 1997).

tannin-containing plants and from three compounds often used as standards, quebracho, cyanidin and delphinidin. There is a more than 30-fold difference in response between the most reactive compound in this assay (delphinidin) and the least reactive compound (quebracho). Based on these relationships, it is apparent that tannin content may be severely underestimated if quebracho is used as the standard (Giner-Chavez et al., 1997). Because condensed tannins from various plant sources differ chemically and in degree of polymerization, the use of external reference standards may be inappropriate (Giner-Chavez et al., 1997).

8. The acid–butanol assay is commonly used to quantify soluble PAs. However, it is now well recognized that some PAs are insoluble in common solvents and, therefore, PAs in plant materials may be underestimated. After PA extraction with 70% acetone, the insoluble fraction was reported to contain between 6 and 20% of the total PAs (Giner-Chavez, 1996). Some of this insoluble PA fraction is fiber-bound and is associated with reduced nitrogen digestibility and increased fecal excretion of nitrogen, fat and water in rats (Bravo et al., 1993; Reed, 1995). The acid–butanol assay has been proposed as a method for estimating the amount of fiber-bound PAs (Reed, 1986, 1995). However, not all bound condensed tannins react quantitatively in the acid–butanol assay, and may thus be underestimated (Makkar et al., 1999).

The butanol–HCl reaction should be used with caution as a quantitative assay. The assay's greatest strength lies in its confirmation of the presence of a polymeric interflavan structure. Hydrolyzable tannins do not react in this assay. Despite the shortcomings outlined above, the acid–butanol assay remains the most commonly used method for determination of PAs in plant tissues.

3.2. *Vanillin assay*

The vanillin method depends on the reaction of vanillin with condensed tannins and the formation of colored complexes. The underlying reaction is shown in Fig. 5. Critical to successful use of this assay are the type of solvent used, the nature and concentration of the acid, the reaction time, temperature, vanillin concentration and type of reference standards used (Scalbert, 1992; Makkar and Becker, 1993; Dalzell and Kerven, 1998; Hagerman, 1998; Sun et al., 1998).

In the vanillin assay, sulfuric acid is a better catalyst than hydrochloric acid at the same normality, but increasing the normality of either acid leads to a higher A_{500} (Scalbert, 1992; Sun et al., 1998). This difference may be attributed to the different water content of the two acids. At the same normality, concentrated HCl contains more water than H_2SO_4 (Sun et al., 1998). At higher water contents, there is a rapid decrease in A_{500} (Bae et al., 1993; Hagerman, 1998; Sun et al., 1998). The use of HCl as a catalyst is also reported to lead to results with low reproducibility (Scalbert, 1992). Temperature also is an important factor that must be controlled in the vanillin assay because an increase of 1.4°C caused an 11% increase in absorbance (Waterman and Mole, 1994). Sun et al. (1998) found a direct relationship between temperature and A_{500}. Their results did not indicate as a severe effect as those of Waterman and Mole (1994), but they cautioned that the reaction should be carried out at 25–30°C to achieve maximum absorbance readings.

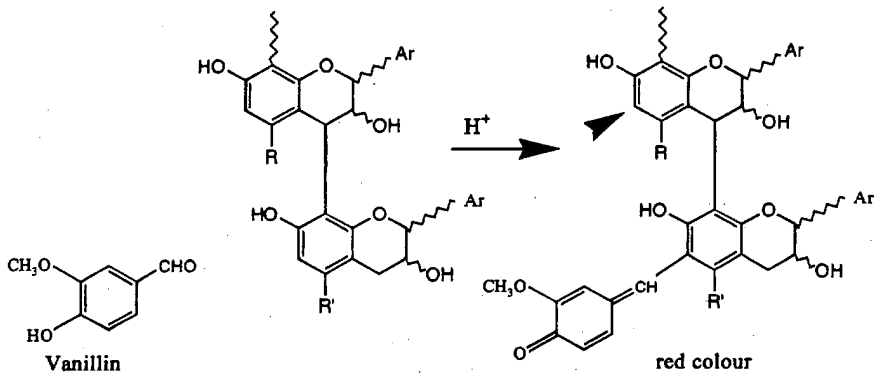


Fig. 5. Chemistry of the vanillin assay for condensed tannins. The arrowhead points to a second potentially reactive site.

Although the most commonly used standard in the vanillin–acid assay is catechin, under normal reaction conditions the catechin monomer has a different reaction rate than related procyanidin polymers (Hagerman, 1998). Due to this problem, internal standards were recommended to express the content of PAs (Sun et al., 1998). Another weakness of the vanillin assay is the lack of specificity for condensed tannins. Any appropriately substituted monomeric flavanol reacts in the assay (Hagerman, 1998). The assay is also insensitive to differences between procyanidins and prodelphinidins in condensed tannins (Waterman and Mole, 1994). A modified vanillin method has been proposed to estimate the molecular weight of condensed tannins instead of using it to quantify total phenolics (Hagerman, 1998; Schofield et al., 1998).

The major problem with the vanillin assay seems to derive from the variable reactivity of the subunits of the tannin polymer. In glacial acetic acid, only terminal units reacted (Butler et al., 1982). Unfortunately, glacial acetic acid is not a good solvent for polymeric PAs. When hydrochloric and sulfuric acids were used as solvents, the color yield per mole from catechin was somewhat greater than that from polymeric PA (Sun et al., 1998). This means that only some of the internal flavanol units in the polymer react with vanillin. Thus structural variations in PA will affect the color yield with vanillin. Many of the problems of the vanillin assay are parallel to those is already noted for the butanol–HCl assay. The complexity and variability of the condensed tannin structures are the root of these analytical difficulties.

3.3. Colorimetric assays for total phenolics

The commonly used methods for determination of total phenolics are the Prussian Blue assay (Price and Butler, 1977) and the Folin–Denis method (Folin and Denis, 1912a,b) later modified by Folin and Ciocalteu (Folin and Ciocalteu, 1927). These methods have been modified numerous times and the version adopted by AOAC (1975) is the one most commonly used today (Waterman and Mole, 1994). These methods entail oxidation–reduction reactions in which the phenolate ion is oxidized while $Fe(CN)_6^{3-}$ ion (Prussian

Blue assay) or phosphotungstic–phosphomolybdic compounds (Folin–Denis assay) are reduced (Graham, 1992; Waterman and Mole, 1994). These assays are not specific for particular groups of phenolic compounds, but rather serve to quantify the total concentration of phenolic hydroxyl groups in the plant extract of interest (Hagerman and Butler, 1994; Waterman and Mole, 1994).

3.3.1. Prussian Blue assay

This is probably the most popular method for quantifying total phenolics because it is simple, rapid and has low interference by non-phenolic compounds (Graham, 1992; Bae et al., 1993; Hagerman et al., 1997). However, there are two important shortcomings of the method (Graham, 1992): (1) formation of a precipitate after short incubation periods; (2) increase in color density with time. Temperature and pH also affect color density (Graham, 1992; Scalbert, 1992). The order in which reagents are added affects the formation of $Fe_4[Fe(CN)_6]_3$ (Prussian Blue).

In one of the most comprehensive studies of this method (Graham, 1992), H_3PO_4 was used to maintain color stability by reducing the pH and complexing excess Fe^{3+}. Precipitation was averted for as long as 60 h by adding gum arabic (1%) to the stabilizing solution. Reagents should be added in the following order: (1) sample; (2) potassium ferricyanide reagent; (3) ferric chloride; (4) the stabilizing solution. A summary of the oxidation–reduction reaction is shown below (Graham, 1992):

$$\text{Polyphenol} + 2Fe(CN)_6^{3-}\,(\text{ferricyanide ion}) \rightarrow \text{oxidized polyphenol} + 2Fe(CN)_6^{4-}\,(\text{ferrocyanide ion}),$$

$$\text{then } 3Fe(CN)_6^{4-}\,(\text{ferrocyanide ion}) + 4Fe^{3+} \rightarrow Fe_4[Fe(CN)_6]_3\,(\text{Prussian blue})$$

Conditions that strongly affect the reactivity of different phenolic hydroxyl groups include reaction time and reagent concentrations. Budini et al. (1980) described a procedure in which non-*ortho* substituted phenols such as orcinol and resorcinol gave no color, whereas those with a single *ortho* or *para* pair of phenolic groups gave approximately the same color yield and gallic acid (having two *ortho*-phenolic groups) gave twice this yield. We have verified the negative response of resorcinol in this assay and have found a similar negative response for phloroglucinol having three *meta*-substituted phenolic groups. Ferulic acid, which contains an *ortho*-activated phenolic hydroxyl group, reacts positively but more slowly than does gallic acid (Schofield, unpublished data). It might be rewarding to explore a combination of the techniques of Graham (1992) and of Budini et al. (1980). This kind of assay would provide a measure of substituent-activated phenolic groups which might correlate well with tannin biological activity.

3.3.2. Folin–Ciocalteu

The Folin–Ciocalteu assay is an improved version of the Folin–Denis method for measuring tyrosine in proteins and all phenols will react. The chromophore produced is a blue phosphotungstic–phosphomolybdic complex of undefined structure and the underlying chemistry of this reaction is not well understood. One of the weaknesses of the Folin–Denis method is the formation of a precipitate that interferes with spectrophotometric measurements (Singleton et al., 1999). The Folin–Ciocalteu reagent was

designed to address this problem by using lithium sulfate (Hagerman et al., 1997; Waterman and Mole, 1994). The method is less prone to interference by non-phenolics than the original method. More details on this method are provided by Singleton et al. (1999) and Waterman and Mole (1994).

3.4. Acid cleavage reactions

The interflavan links in most condensed tannins may be broken under acid conditions. The acid–butanol colorimetric assay discussed above is one example of acid cleavage. Other examples include the acid-catalyzed condensation reactions with benzyl mercaptan and phloroglucinol (Fig. 6).

3.4.1. Thiolysis

The reaction occurring when condensed tannins are heated in the presence of acid and benzyl mercaptan (also known as toluene-α-thiol, phenylmethane thiol) is shown in Fig. 6. The chain ending unit at the bottom (with a free 4 position) is released as an unsubstituted flavanol, whereas all internal units appear as benzyl thioethers. In principle, this would permit determination of both chain length and composition of a tannin when thiolysis products are analyzed by HPLC. Problems arise if the material analyzed is heterogeneous or if thiolysis is incomplete. Matthews et al. (1997) studied bark tannins from *Picea sitchensis* and performed thiolysis for 4.5 h at 105°C with 1.4% acetic acid. Under these conditions, the maximum thiolysis yield was 63%. However, Labarbe et al. (1999), using more highly purified grape PAs, reported thiolysis yields of 88% using 0.1 M HCl at 90°C for 2 min. It is not clear to what extent structural differences or differences in reaction conditions contributed to these different outcomes.

3.4.2. Phloroglucinol degradation

Phloroglucinol reacts with condensed tannins similarly to benzyl mercaptan (Fig. 6). Matthews et al. (1997) found that a large portion of their bark tannin did not react with

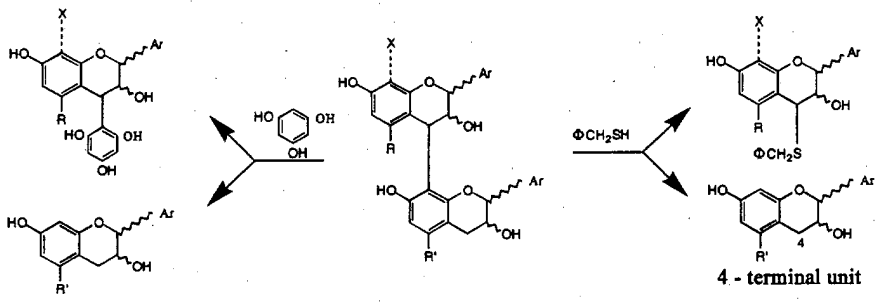


Fig. 6. Chemistry of the thiolysis and phloroglucinol condensation reactions. Only the original terminal unit is released without an added substituent. The total reaction proceeds in many steps and cleavage does not necessarily start at the terminus.

phloroglucinol and that yields of adduct were between 3 and 10%. Given this finding, it would seem that thiolytic degradation is to be preferred over the phloroglucinol reaction despite the unpleasant smell and lachrymatory characteristics of benzyl mercaptan.

We should note that profisetinidins and prorobinetinidins (Fig. 1) will behave abnormally in all acid cleavage reactions, including the butanol–HCl reaction, because the interflavan bond is more stable to acid in the absence of a 5-OH group than is the case in the more common PAs.

3.5. Gravimetric methods

Two gravimetric approaches for quantification of phenolic compounds in plant materials have been described by Reed et al. (1985) and Makkar et al. (1993). The method by Reed et al. uses trivalent ytterbium to precipitate soluble phenolics while the one by Makkar et al. measures tannin binding to insoluble polyvinylpyrrolidone. A primary advantage of gravimetric methods is that they do not require standards. However, they are less sensitive than the common colorimetric methods (Makkar et al., 1993). For example, the method of Makkar et al. (1993) requires about 30 mg of PVP to bind 2 mg of tannic acid completely. The percentage weight change in insoluble PVP is thus quite small.

3.5.1. Ytterbium precipitation assay

This method was used by Giner-Chavez et al. (1997) to precipitate condensed tannins for later use as standards in the acid–butanol assay. The amount of polyphenols is determined by deducting the amount of ash (ytterbium oxide) after ashing the dry precipitated complex. To validate the ytterbium method, the tannins isolated by the trivalent ytterbium method were compared to those purified with the commonly used Sephadex LH 20 (Hagerman, 1998). When the two extracts were used as internal standards, the resulting predictions of tannin contents of the forages were similar. The ytterbium extraction method requires less time and is less expensive than the Sephadex procedure (Giner-Chavez et al., 1997). The utility of the ytterbium method was questioned because some model phenolics like rutin were not precipitated by ytterbium (Lowry and Sumpter, 1990). However, most model phenolics are precipitated if the molar ratio of the phenolics to the ytterbium is controlled (Giner-Chavez, 1996). As an aside, we note that rutin contains only a single flavanoid unit and is not a true tannin.

3.6. Assays based on enzyme activity changes

Tannins inhibit the activity of many different enzymes (Goldstein and Swain, 1965). Tannin assay methods based on this property are attractive because they reflect what may be an important biological mode of action. In addition, these methods can be used to explore tannin/protein interactions in solution. Variables include choice of enzyme, pH, ionic strength, temperature and time. The experimental design may depend on the nature of the question asked. If emphasis is placed on how given tannins affect a specific biological process, then the enzyme studied must reflect that process. Tannins that frequently interfere with digestion and digestive enzymes such as trypsin or amylase

would be natural choices for this kind of study. If, on the other hand, the main interest is to compare the relative protein-binding properties of a range of tannins, then the choice of enzyme becomes more open and factors of convenience can influence this choice. Thus, the enzyme inhibition caused by different accessions of *Desmodium ovalifolium* might be explored using, say, urease as a target. There are many publications on enzyme inhibition by tannins; the following survey covers only a restricted sample.

3.6.1. Digestive enzymes

These enzymes act on polymeric substrates such as proteins, carbohydrates and nucleic acids. Tannins could intervene by binding to either the enzyme (e.g. trypsin) or the substrate (e.g. leaf protein) or to both (Mole and Waterman, 1987). One way to simplify this situation is to remove the substrate as a potential target by using a low molecular weight analog such as benzoylarginine-*p*-nitroanilide (BAPNA) for trypsin or *p*-nitrophenylphosphate (PNPP) for phosphatase. An alternative approach is to conduct the assay in two stages. The enzyme is first pre-incubated with tannin in the absence of substrate then the substrate is added and the mixture incubated further.

3.6.1.1. β-Glucosidase. Inhibition by a hydrolysable tannin (tannic acid) and wattle condensed tannin was incomplete and could be reversed by non-ionic detergents, or by polyvinylpyrrolidine (PVP) and polyethylene glycol (PEG) (Goldstein and Swain, 1965). Although this study did not purport to use inhibition of β-glucosidase as an assay method, it has been influential in the design of other assays.

3.6.1.2. Trypsin. Studies of the inhibition of trypsin by tannins from pears, lentils and cocoa were done using BAPNA as substrate (Quesada et al., 1995). Trypsin and BAPNA were also applied in a microtiter plate format to compare the tannin-binding capacities of salivary proteins (Fickel et al., 1999).

3.6.1.3. Amylase. Inhibition of this enzyme by various tannins was reported by Quesada et al. (1995) using a starch/iodine assay. Linear inhibition occurred over the range 0–40 μg/ml of sorghum condensed tannins.

3.6.1.4. Cellulase. Tannins from *Lespedeza cuneata* inhibited the activity of a fungal cellulase (from *Trichoderma viride*) in a non-competitive manner. This inhibition could be reversed by adding other proteins as alternative targets for the tannin (Petersen and Hill, 1991).

3.6.1.5. Alkaline phosphatase. An interesting variation on the tannin/enzyme theme was reported by Ittah (1991) who found that persimmon tannins could bind alkaline phosphatase to microtiter plates coated with trace amounts of bovine serum albumin (BSA). The bound phosphatase retained its activity and the amount bound reflected the amount of tannin applied. The enzyme could then be assayed using PNPP. The plate format allowed many samples to be assayed simultaneously and automatically. This technique has been modified for analysis of tannins in grapes and red wine (Adams and Harbertson, 1999).

In summary, the advantages of enzyme techniques for condensed (and hydrolysable) tannin assay are: (1) high sensitivity because the enzyme acts as an amplifier; (2) the process measured may be biologically relevant; (3) interactions occur in solution at low concentrations and do not necessarily depend on precipitation events. Disadvantages include the problem, common to many assays, that the substrate and assay conditions may not resemble processes of biological interest.

3.7. Protein precipitation assays

Tannins are, by definition, protein-binding and precipitating agents and tannin assays based on protein precipitation have long been popular. Persons contemplating the use of this kind of assay should consult the extended discussion of the topic by Waterman and Mole (1994). The most convenient form of this assay uses a protein, normally BSA, dyed with Remazol Brilliant Blue (Asquith and Butler, 1985). Tannin and dyed protein are mixed under defined conditions of pH and ionic strength and allowed to precipitate. After centrifugation, the pellet is redissolved in an alkaline SDS buffer for measurement of the dyed protein content. Protein precipitation can also be followed in agarose plates (Hagerman, 1987). Although only semi-quantitative, this plate method does not require a dye-labeled protein and permits the simultaneous analysis of many samples.

3.8. Other precipitation assays

Tannins form insoluble complexes with PEG and with many dyes such as Methylene Blue (Okuda et al., 1985). Work on Methylene Blue binding and precipitation is currently in progress (Schofield and Pell). Early results have confirmed the observations of Okuda et al. (1985) that only tannins containing galloyl or related structures react strongly with this dye.

3.8.1. PEG binding assay

An in situ method for determination of tannins in plant samples without extraction using ^{14}C-labeled PEG has been reported (Silanikove et al., 1996a) Polyethylene glycol 4000 (PEG), which binds to a wide range of hydrolysable and condensed tannins, formed a stable PEG–tannin complex over a pH range from 2 to 8.5 (Jones, 1965) and this tannin–PEG complex was insoluble in boiling water, neutral and acid detergents and many organic solvents (Jones, 1965; Jones and Mangan, 1977). The method is reportedly useful for plant materials containing strong tannin–protein complexes in which extraction would give low tannin yields. The PEG can disrupt these complexes because its affinity for tannin exceeds that of protein. The method has been proposed as a tool for predicting adverse effects of tannins on ruminal degradation (Silanikove et al., 1996b). The primary disadvantages of this method are safety issues and costs of working with and disposing of radioactive materials.

3.9. Analysis of PA with high performance liquid chromatography (HPLC)

Condensed tannins can be quantified using HPLC. Both normal-phase and reversed-phase columns have been applied (Cheynier et al., 1999; Lazarus et al., 1999; Waterhouse et al., 1999). Reversed-phase HPLC has been used for separation of lower molecular

weight condensed tannins but the order of elution is not related to the degree of polymerization (Cheynier et al., 1999). Separation of larger polymers (≥tetramers) with this method is not possible. The presence of many isomers with similar polarity, results in overlapping retention times (Lazarus et al., 1999; Waterhouse et al., 1999). Normal-phase HPLC was used to separate condensed tannin oligomers and polymers (Lazarus et al., 1999) from various food plant products. The order of elution increased with increase in degree of polymerization.

Various detection methods have been applied in conjunction with HPLC for PA determination. Ultraviolet (UV) detection is the most commonly used (Waterman and Mole, 1994). However, this method is not specific for PA in the presence of other polyphenols (Lazarus et al., 1999). Alternative methods include electrochemical detection and fluorescence detection (Waterman and Mole, 1994; Lazarus et al., 1999). Structural information for identification of PA oligomers can be obtained using mass spectrometry (MS), nuclear magnetic resonance (NMR) and chemical hydrolysis (Hammerstone et al., 1999).

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and fast-atom bombardment MS (FAB-MS) were used to characterize unripe apple catechin-based condensed tannin polymers larger than the decamer (Ohnishi-Kameyama et al., 1997). The use of HPLC coupled with various detection methods will undoubtedly continue to increase and will play a major role in the elucidation of the complexity of both condensed and hydrolysable tannins.

3.10. Inhibition of microbial growth

Inhibition of microbes by tannins leads to reduced fiber digestion in ruminants and to decreased infection of plants by plant pathogens. The impacts of tannins on microbes can be assessed either by comparisons of microbial growth measured in tubes with and without tannins (Nelson et al., 1997) or by measuring differences in substrate disappearance. In vitro fermentation data can be used to assess the inhibition of microbes by tannins (Giner-Chavez, 1996). In this method, tannin-containing plant samples are treated with neutral detergent to remove non-fiber-bound tannins. Differences in fiber digestibility between extracted and untreated samples provide a relative index of inhibition of microbial fiber digestion by tannins. A third approach is to measure binding of tannins to bacteria, an assay similar to the protein-binding assays discussed previously (Jones et al., 1994; Nelson et al., 1997). In the bacterial binding assay, tannins are added to bacterial cultures grown without tannins in the medium, and the cells are centrifuged to form a pellet. After removal of the supernatant and rinsing with buffer, the cells are repelleted and analyzed for tannins (Nelson et al., 1997).

4. Tannins: speculations on structure–activity relationships

4.1. Structure–activity

The structural distinction between hydrolysable and condensed tannins is founded on two features: the presence of ester-linked gallic or ellagic acids in the former; the interflavan C–C linkage in the latter. As more information became available on condensed tannin structures, the clarity of this distinction began to fade. Condensed tannins may also contain gallic acid esterified to the 3-OH of ring C (Fig. 1). What role might these esterified gallates play?

The key feature that gives tannins their characteristic properties seems to be an unusually high local concentration of *ortho*-phenolic hydroxyl groups (Haslam, 1974). In hydrolysable tannins, these are associated mainly with gallic or ellagic acid residues. In condensed tannins (procyanidins, prodelphinidins) ring B phenolic hydroxyls play this role (esterified gallate may also contribute). We support the suggestion of Haslam (1974) that the relative biological activity of tannins may be dictated, at least in part, by the molar content of these *ortho*-phenolic groups. Their spatial concentration also may be important. Hydrolysable tannins are, in general, more potent biological agents per unit mass than are condensed tannins (Haslam, 1974) and the protein precipitating capacity of hydrolysable tannins is directly related to the number of galloyl groups (Beart et al., 1985). Compare now the structure of pentagalloyl glucose (PGG, FW = 942 Da), a starting point for elaboration of tannic acid (Mueller-Harvey, 2001), with that of a grape tannin having three flavanol subunits (Labarbe et al., 1999) (cat_3, FW ~ 860). PGG has 15 *ortho*-phenolic groups while cat_3 has six. We might therefore speculate that condensed tannins with higher levels of esterified gallate would be more biologically active than those with less gallate. Cheynier et al. (1999) found that grape seed condensed tannins contained about 20% esterified gallate, whereas grape skin tannins had much less (about 2%). Studies on the relative biological activity of grape seed and grape skin tannins have not yet been reported.

4.2. Choice of assay

In choosing a tannin assay one must be clear about the question(s) being asked. These questions, in order of increasing detail, include:

1. Are tannins present — yes or no?
   Spot tests such as the butanol–HCl and total phenolic assays can give a quick but not certain answer to this question.
2. What is the amount of material with tannin-like properties?
   Hazards associated with quantitative tannin estimation, especially using the butanol–HCl reaction, have already been discussed. Choice of the appropriate standard is most critical for this question. Internal standards, extracted and purified from the material under investigation, will give more reliable results than external standards. Any of the usual colorimetric or precipitation methods can be used.
3. What is the biological activity of this material?
   This is a difficult question to answer. Possible assays include protein precipitation and enzyme inhibition methods. See the appropriate sections of this review for details. Enzyme inhibition assays would seem to be one natural choice because they provide a sensitive measure of tannin–protein interactions and may reflect a biologically significant mode of action. Among the colorimetric chemical assays, the total phenolics method would seem to have some advantages over alternative methods

Table 2
A summary of available assay methods for condensed tannins

Assay name	Assay type	Pro	Con	Comments
Acid–butanol (AB)	Chemical, colorimetric	Specific for condensed tannins	Requires internal standard. Color yield varies with tannin structure	Classical method. Not well suited to quantification
Vanillin	Chemical, colorimetric	Specific for *meta*-diphenols	Same as AB. Simple diphenols also react	
Prussian Blue	Chemical, colorimetric	General test for phenols	Depending on conditions, all phenols can react. Reducing agents react also	Gives best correlation with biological activity
Folin–Ciocalteu	Chemical, colorimetric		Complex chemistry. All phenols react	Prussian Blue method gives better reading of total phenolic content
Thiolysis	Chemical, needs HPLC	Good for structure determination	Requires pure tannin	Benzyl mercaptan is unpleasant to handle
Phloroglucinol	Chemical, needs HPLC		Product yields tend to be low	Thiolysis gives better yields of cleavage products
Ytterbium precipitation	Gravimetric	No standard needed	Yield may vary with Yb:tannin ratio. Sample must be ashed	Can be used to prepare standards for colorimetric analysis
Enzyme assay	Enzymatic inhibition	Gives a more biological evaluation	Some enzymes much more susceptible than others	Does not rely on protein precipitation
Protein precipitation	Precipitation	Reflects a biologically important process	Results depend on many variables such as choice of protein	Can be done in agar plates (Hagerman)
PEG precipitation	Precipitation	Can assay protein-bound tannin	Requires ^{14}C-PEG	
HPLC	HPLC	For polymers up to 7–8 units long	Some condensed tannins bind irreversibly	Better reserved for structural studies
Microbial growth inhibition	Toxicity	A good biological assay	Choice of bacteria and medium composition will affect results	Requires relatively high tannin levels because of competing tannin-binding agents

because it can be designed to reflect only the *ortho*-di or tri-phenolic content of the tannin and there is some evidence that this *ortho*-phenolic content is correlated with biological activity.

4. Are the tannins of the hydrolysable or condensed type (or both)?

The butanol–HCl and vanillin reactions are useful diagnostic tools for condensed tannins. To detect hydrolysable tannins, the rhodanine assay of Inoue and Hagerman (1988) for gallate esters may serve — see the accompanying paper by Mueller-Harvey in this volume.

5. What are the molecular sizes/structures of tannins present?

Assuming that one has a homogeneous starting material, the physicochemical methods employing thiolysis and gas chromatography/MS can give detailed information about tannin structure.

A brief summary of the analysis techniques discussed in this paper appears in Table 2. The most useful advice when looking for a tannin assay is to do more than one type of assay, bearing in mind the reasons for gathering assay data.

5. Resources

The following references contain useful discussions of many of the techniques mentioned in this review: Salunkhe et al. (1989), Waterman and Mole (1994), Hagerman et al. (1997), Hagerman (1998), Santos-Buelga and Scalbert (2000).

References

Adams, D.O., Harbertson, J.F., 1999. Use of alkaline phosphatase for the analysis of tannins in grapes and red wines. Am. J. Enol. Vitic. 50, 247–252.

Aerts, R.J., Barry, T.N., McNabb, W.C., 1999. Polyphenols and agriculture: beneficial effects of proanthocyanidins in forages. Agric. Ecosyst. Environ. 75, 1–12.

AOAC, 1975. Official Methods of Analysis, 12th Edition. AOAC, Washington, DC.

Asquith, T.N., Butler, L.G., 1985. Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannin. J. Chem. Ecol. 11, 1535–1544.

Bae, H.-D., McAllister, T.A., Müir, A.D., Yanke, J., Bassendowski, K.A., Cheng, K.-J., 1993. Selection of a method of condensed tannin analysis for studies with rumen bacteria. J. Agric. Food Chem. 41, 1256–1260.

Beart, J.E., Lilley, T.H., Haslam, E., 1985. Plant polyphenols — secondary metabolism and chemical defence: some observations. Phytochemistry 24, 33–38.

Bravo, L., Mañas, E., Saura-Calixto, S., 1993. Dietary non-extractable condensed tannins as indigestible compounds: effects on faecal weight, and protein and fat excretion. J. Sci. Food Agric. 63, 63–68.

Budini, R., Tonelli, D., Girotti, S., 1980. Analysis of total phenols using the Prussian blue method. J. Agric. Food Chem. 28, 1236–1238.

Butler, L.G., Price, M.L., Brotherton, J.E., 1982. Vanillin assay for proanthocyanidins (condensed tannins): modification of the solvent for estimation of the degree of polymerization. J. Agric. Food Chem. 30, 1087–1089.

Cheynier, V., Souquet, J.-M., Le Roux, E., Guyot, S., Rigaud, J., 1999. Size separation of condensed tannins by normal-phase high-performance liquid chromatography. Meth. Enzymol. 299, 178–184.

Dalzell, S.A., Kerven, G.L., 1998. A rapid method for the measurement of *Leucaena* spp. proanthocyanidins by the proanthocyanidin (butanol/HCl) assay. J. Sci. Food Agric. 78, 405–416.

Fickel, J., Pitra, C., Joest, B.A., Hofmann, R.R., 1999. A novel method to evaluate the relative tannin-binding capacities of salivary proteins. Comp. Biochem. Physiol., Part C 122, 225–229.
Folin, O., Ciocalteu, V., 1927. On tyrosine and tryptophan determinations in proteins. J. Biol. Chem. 27, 627–650.
Folin, O., Denis, W., 1912a. Tyrosine in proteins as determined by a new colorimetric method. J. Biol. Chem. 12, 245–251.
Folin, O., Denis, W., 1912b. On phosphotungstic–phosphomolybdic compounds as colour reagents. J. Biol. Chem. 12, 239–243.
Foo, L.Y., Lu, Y., McNabb, W.C., Waghorn, G.C., Ulyatt, M.J., 1997. Proanthocyanidins from *Lotus pedunculatus*. Phytochemistry 45, 1689–1696.
Giner-Chavez, B., 1996. Condensed tannins in tropical forages. Thesis. Cornell University, Ithaca, NY.
Giner-Chavez, B., Van Soest, P.J., Robertson, J.B., Pell, A.N., Lascano, C.E., Reed, J.D., 1997. A method for isolating condensed tannins from crude plant extracts with trivalent ytterbium. J. Sci. Food Agric. 74, 359–368.
Goldstein, J.L., Swain, T., 1965. The inhibition of enzymes by tannins. Phytochemistry 4, 185–192.
Graham, H.D., 1992. Stabilization of the Prussian blue color in the determination of polyphenols. J. Agric. Food Chem. 40, 801–805.
Hagerman, A.E., 1987. Radial diffusion method for determining tannin in plant extracts. J. Chem. Ecol. 13, 437–449.
Hagerman, A.E., 1992. Tannin–protein interactions. In: Ho, C.-T., Lee, C.Y., Huang, M.-T. (Eds.), Phenolic Compounds in Food and Their Effects on Health. 1. Analysis, Occurrence and Chemistry. American Chemical Society, Washington, DC, pp. 236–247 (Chapter 19).
Hagerman, A.E., 1998. Tannin Analysis. http://miavx1.edu/~hagermae/.
Hagerman, A.E., Butler, L.G., 1989. Choosing appropriate methods and standards for assaying tannin. J. Chem. Ecol. 15, 1795–1810.
Hagerman, A.E., Butler, L.G., 1994. Assay of condensed tannins or flavonoid oligomers and related flavonoids in plants. Meth. Enzymol. 234, 429–437.
Hagerman, A.E., Zhao, Y., Johnson, S., 1997. Methods for determination of condensed and hydrolyzable tannins. In: Shahidi, F. (Ed.), Antinutrients and Phytochemicals in Food. American Chemical Society, Washington, DC.
Hagerman, A.E., Rice, M.E., Ritchard, N.T., 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4->8) catechin (procyanidin). J. Agric. Food Chem. 46, 2590–2595.
Hammerstone, J.F., Lazarus, S.A., Mitchell, A.E., Rucker, R., Schmitz, H.H., 1999. Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. J. Agric. Food Chem. 47, 490–496.
Harborne, J., 1998. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. Chapman & Hall, London.
Harborne, J.B., Baxter, H., 1999. The Handbook of Natural Flavonoids. Wiley, Chichester, UK.
Haslam, E., 1974. Polyphenol–protein interactions. Biochem. J. 139, 285–288.
Haslam, E., 1989. Plant Polyphenols. Cambridge University Press, Cambridge.
Hemingway, R.W., 1989. Reactions at the interflavanoid bond of proanthocyanidins. In: Hemingway, R.W., Karchesy, J.J. (Eds.), Chemistry and Significance of Condensed Tannins. Plenum Press, New York.
Inoue, K.H., Hagerman, A.E., 1988. Determination of gallotannin with rhodanine. Anal. Biochem. 169, 363–369.
Ittah, Y., 1991. Titration of tannin via alkaline phosphatase activity. Anal. Biochem. 192, 277–280.
Jones, D.E., 1965. Banana tannin and its reaction with polyethylene glycols. Nature 205, 299–300.
Jones, W.T., Mangan, J.L., 1977. Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* Scop.) with fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. J. Sci. Food Agric. 28, 126–136.
Jones, G.A., McAllister, T.A., Muir, A.D., Cheng, K.-J., 1994. Effects of sainfoin (*Onobrychis viciifolia* Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. Appl. Environ. Microbiol. 60, 1374–1378.
Labarbe, L., Cheynier, V., Broussard, F., Souquet, J.-M., Moutounet, M., 1999. Quantitative fractionation of grape proanthocyanidins according to their degree of polymerization. J. Agric. Food Chem. 47, 2719–2723.

Lazarus, S.A., Adamson, G.E., Hammerstone, J.F., Schmitz, H.H., 1999. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. J. Agric. Food Chem. 47, 3693–3701.
Lowry, J.B., Sumpter, E.A., 1990. Problems with ytterbium precipitation as a method for determination of plant phenolics. J. Sci. Food Agric. 52, 287–288.
Makkar, H.P.S., Becker, K., 1993. Vanillin–HCl method for condensed tannins: effect of organic solvents used for extraction of tannins. J. Chem. Ecol. 19, 613–621.
Makkar, H.P.S., Blümmel, M., Borowy, N.K., Becker, K., 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. 61, 161–165.
Makkar, H.P.S., Gamble, G., Becker, K., 1999. Limitation of the butanol–hydrocloric acid–iron assay for bound condensed tannins. Food Chem. 66, 129–133.
Matthews, S., Mila, I., Scalbert, A., Pollet, B., Lapierre, C., Hervé du Penhoat, C.L.M., Rolando, C., Donnelly, D.M.X., 1997. Method for estimation of proanthocyanidins based on their acid depolymerization in the presence of nucleophiles. J. Agric. Food Chem. 45, 1195–1201.
Mole, S., Waterman, P.G., 1987. Tannic acid and proteolytic enzymes: enzyme inhibition or substrate deprivation. Phytochemistry 26, 99–102.
Mueller-Harvey, I., 2001. Analysis of hydrolysable tannins. Anim. Feed Sci. Technol. 91, 3–20.
Mueller-Harvey, I., McAllan, A.B., 1992. Tannins: their biochemistry and nutritional properties. Adv. Plant Cell Biochem. Biotechnol. 1, 151–217.
Naczk, M., Oickle, D., Pink, D., Shahidi, F., 1996. Protein precipitating capacity of crude canola tannins: effect of pH, tannin and protein concentrations. J. Agric. Food Chem. 44, 2144–2148.
Nelson, K.E., Pell, A.N., Doane, P.H., Giner-Chavez, B., Schofield, P., 1997. Chemical and biological assays to evaluate bacterial inhibition by tannins. J. Chem. Ecol. 23, 1175–1194.
Ohnishi-Kameyama, M., Yanagida, A., Kanda, T., Nagata, T., 1997. Identification of catechin oligomers from apple (*Malus pumila* cv. Fuji) in matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and fast-atom bombardment mass spectrometry. Rapid Commun. Mass Spectrom. 11, 31–36.
Okuda, T., Mori, K., Hatano, T., 1985. Relationship of the structures of tannins to the binding activities with hemoglobin and methylene blue. Chem. Pharm. Bull. 33, 1424–1433.
Petersen, J.C., Hill, N.S., 1991. Enzyme inhibition by Sericea Lespedeza tannins and the use of supplements to restore activity. Crop Sci. 31, 827–832.
Porter, L.J., Hrstich, L.N., Chan, B.G., 1986. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. Phytochemistry 1, 223–230.
Price, M.L., Butler, L.G., 1977. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. J. Agric. Food Chem. 25, 1268–1277.
Prieur, C., Rigaud, J., Cheynier, V., Moutounet, M., 1994. Oligomeric and polymeric procyanidins from grape seeds. Phytochemistry 36, 781–784.
Quesada, C., Bartolomé, B., Nieto, O., Gómez-Cordovés, C., Hernández, T., Estrella, I., 1995. Phenolic inhibitors of α-amylase and trypsin enzymes by extracts from pears, lentils and cocoa. J. Food Prot. 59, 185–192.
Reed, J.D., 1986. Relationships among soluble phenolics, insoluble proanthocyanidins and fiber in East African browse species. J. Range Mgmt. 39, 5–7.
Reed, J.D., 1995. Nutritional toxicology of tannins and related polyphenols in forage legumes. J. Anim. Sci. 73, 1516–1528.
Reed, J.D., McDowell, R.E., Van Soest, P.J., Horvath, P.J., 1982. Condensed tannins: a factor limiting the use of cassava forage. J. Sci. Food Agric. 33, 213–220.
Reed, J.D., Horvath, P.J., Allen, M.S., Van Soest, P.J., 1985. Gravimetric determination of soluble phenolics including tannins from leaves by precipitation with trivalent ytterbium. J. Sci. Food Agric. 36, 255–261.
Salunkhe, D.K., Chavan, J.K., Kadam, S.S., 1989. Dietary Tannins: Consequences and Remedies. CRC Press, Boca Raton, FL.
Santos-Buelga, C., Scalbert, A., 2000. Proanthocyanidins and tannin-like compounds — nature, occurrence, dietary intake and effects on nutrition and health. J. Sci. Food Agric. 80, 1094–1117.
Scalbert, A., 1991. Antimicrobial properties of tannins. Phytochemistry 30, 3875–3883.

Scalbert, A., 1992. Quantitative methods for the estimation of tannins in plant tissues. In: Hemingway, R.W., Laks, P.E. (Eds.), Plant Polyphenols: Synthesis, Properties, Significance. Plenum Press, New York.

Schofield, J.A., Hagerman, A.E., Harold, A., 1998. Loss of tannins and other phenolics from willow leaf litter. J. Chem. Ecol. 24, 1409–1421.

Silanikove, N., Gilboa, N., Nir, I., Perevolotsky, A., Nitsan, Z., 1996a. Effect of a daily supplementation of polyethylene glycol on intake and digestion of tannin-containing leaves (*Quercus calliprinos*, *Pistacia lentiscus*, and *Ceratonia siliqua*) by goats. J. Agric. Food Chem. 44, 199–205.

Silanikove, N., Shinder, D., Gilboa, N., Eyal, M., Nitsan, Z., 1996b. Binding of poly(ethylene glycol) to samples of forage plants as an assay of tannins and their negative effects on ruminal degradation. J. Agric. Food Chem. 44, 3230–3234.

Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Meth. Enzymol. 299, 152–178.

Sun, B., Ricardo-da-Silva, J.M., Spranger, I., 1998. Critical factors of vanillin assay for catechins and proanthocyanidins. J. Agric. Food Chem. 46, 4267–4274.

Terrill, T.H., Rowan, A.M., Douglas, G.B., Barry, T.N., 1992. Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. J. Sci. Food Agric. 58, 321–329.

Van Acker, S.A.B.E., Plemper van Balen, G.P., van den Berg, D.J., Bast, A., van der Vijgh, W.J.F., 1998. Influence of iron chelation on the antioxidant activity of flavonoids. Biochem. Pharmacol. 56, 935–943.

van Hoven, W., Furstenburg, D., 1992. The use of purified condensed tannin as a reference in determining its influence on rumen fermentation. Comp. Biochem. Physiol. A 101, 381–385.

Waterhouse, A.L., Price, S.F., McCord, J.D., 1999. Reversed-phase high-performance liquid chromatography methods for analysis of wine polyphenols. Meth. Enzymol. 299, 113–121.

Waterman, P.G., Mole, S., 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications, Oxford.

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Abstract

Acacia angusti adaptation at lev determined that r used in bioassays 7.5% *A. angustis* compared to rat *angustisstima* leav toxicity symptor transformed by oven drying. Var 7.5% *A. angustis* gross pathologic factor(s) milled added to rat diet reduced in rats fe (8.8 and 3.1 g/d) wet weight and/ and increased fractionation of soluble fractions

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LEUCOCYANIDIN REDUCTASE ACTIVITY AND ACCUMULATION OF PROANTHOCYANIDINS IN DEVELOPING LEGUME TISSUES[1]

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Proanthocyanidin (PA) and anthocyanin accumulation and location in developing leaves, flowers, and seeds of the legumes *Medicago sativa, Lotus japonicus, Lotus uliginosus, Hedysarum sulfurescens,* and *Robinia pseudacacia* were investigated by quantitative measurements and by histological analysis after staining with 1% vanillin/HCl, butanol/HCl, or 50% HCl. *M. sativa* leaves and flowers, *L. japonicus* leaves, and *R. pseudacacia* flowers do not contain PAs, but seeds of all investigated species contain PAs. Anthocyanins are absent in the seed coats of all five species and in leaves of *L. japonicus.* PA content generally increases as a function of development in leaves, but declines in flowers. With the exception of *H. sulfurescens,* flower PAs are synthesized in the parenchyma cells of the standard petal, while anthocyanins are located in the neighboring epidermal cells. Leucocyanidin reductase (LCR) catalyzes the conversion of 2,3-*trans*-3,4-*cis*-leucocyanidin to (+)-catechin and is the first enzyme in the PA-specific pathway. LCR activity was only detected in PA-containing tissues and generally declined during tissue development.

Key words: anthocyanins; *Hedysarum sulfurescens*; Leguminosae; leucocyanidin reductase; *Lotus japonicus*; *Lotus uliginosus*; *Medicago sativa*; proanthocyanidins; *Robinia pseudacacia.*

Flavonoids belong to a highly diversified class of compounds originating from the phenylpropanoid and acetogenic pathway (Dooner, Robbins, and Jørgensen, 1991). While some flavonoids accumulate in a number of ferns and gymnosperms, an extraordinary multiplicity of variants has evolved among the angiosperms, the variation being due to different substituents on the flavan skeleton (Harborne, 1988). An increasing number of flavonoids can be assigned important biological functions (Dakora, 1995). Among these is the induction of *nod*-gene transcription in *Rhizobium* by certain flavonoids released from *Phaseolus vulgaris* (Hungria, Joseph, and Phillips, 1991) and *Medicago sativa* (Peters, Frost, and Long, 1986; Maxwell et al., 1989; Hartwig, Joseph, and Phillips, 1991), which in turn promotes the formation of root nodules and N_2 fixation. Flavonols have also turned out to be essential for pollen germination in maize (Deboo, Albertsen, and Taylor, 1995) and *Petunia* (Ylstra et al., 1994).

Proanthocyanidins (PAs), also known as condensed tannins, are flavonoid polymers that accumulate in various tissues of many plant species. An important property of the PAs is their ability to bind and precipitate proteins, which derives from the fact that they are multidentate ligands able to bind simultaneously at more than one point (Spencer et al., 1988). This property may lead to the formation of colloidal haze in beer if no stabilizing treatment is performed (Erdal, 1986), a problem that can be circumvented by using PA-free barley as raw material (Wettstein et al., 1977). When present in forage legumes, such as *Lotus corniculatus* and *Onobrychis viciifolia,* the ability of PAs to react with dietary proteins and to form stable complexes may have profound effects on animal nutrition. This property is considered the main reason for their action as rumen antibloat agents, since the complexes inhibit the formation of a stable foam (Jones, Broadhurst, and Lyttleton, 1976). Furthermore, the dietary plant proteins are protected against bacterial deamination in the rumen, providing a protein by-pass mechanism that results in increased duodenal absorption of amino acids (McNabb et al., 1993; Tanner, Moore, and Larkin, 1994). PAs and monomeric flavan-3-ols have recently been shown to have antioxidant properties (Hara, 1994) and in some plants the presence of PAs has been correlated with insect resistance (Chan, Waiss, and Lukefahr, 1978; Hedin and Waage, 1986).

Flavonoid composition is an obvious target for value-added improvement of crop plants, but exact knowledge of the constituents of PAs identified (Koupai-Abyazani, McCallum, and Bohm, 1992) and their biosynthesis is required for successful modification of the pathway. The genetic control of the biosynthesis of flavonoid monomers such as anthocyanins and isoflavonoids has been characterized to a considerable extent (Dooner, Robbins, and Jørgensen, 1991; Jende-Strid, 1993; Dixon and Paiva, 1995; Holton and Cornish, 1995), including the cloning of several genes. With genetic transformation becoming a routine technique for a number of crop plants, the ability to alter PA content and composition for biotech-

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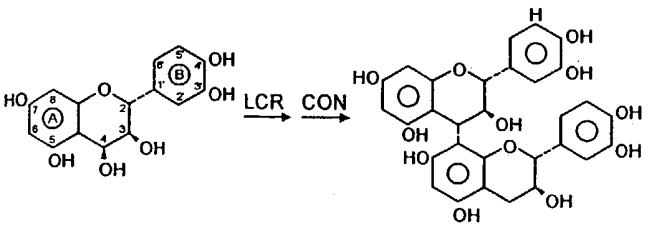
nological purposes is an achievable goal. For example, in root cultures of the legume *Lotus corniculatus,* PA synthesis was inhibited by antisense constructs based on the gene encoding chalcone synthase (CHS) (Robbins, Carron, and Morris, 1992). However, so far only genes encoding enzymes in the biosynthesis of PA prior to leucocyanidin reduction have been isolated (Kristiansen and Rohde, 1991; Meldgaard, 1992).

PAs are produced by condensation of flavan-3-ol units with flavan-3,4-diols (Kristiansen, 1984, 1986; Stafford, 1990). For example, the prototype procyanidin B-3 is formed by reduction of (+)-2R,3S-*trans*-3R,4R-*cis*-leucocyanidin (dihydroxyl-flavanol) to (+)-catechin with the enzyme leucocyanidin reductase (LCR) and subsequent condensation of catechin with leucocyanidin by a putative condensing enzyme (CON) as shown in Scheme 1.



Scheme 1. Biosynthesis of procyanidin B-3 from (+)-2R, 3S-*trans*-3R, 4R-*cis*-leucocyanidin. LCR = leucocyanidin reductase; CON = condensing enzyme.

Leucocyanidin reductase marks the branch point between the anthocyanin- and PA-specific pathway. This enzyme uses dihydroxyl-flavanol to synthesize catechin and procyanidin-containing PAs and is specific for the formation of proanthocyanidins (Tanner and Kristiansen, 1993).

So far, it has not been possible to purify either this enzyme or an enzyme catalyzing the subsequent condensing step, nor has it been possible to isolate the corresponding structural genes. Mutants preventing the synthesis of PAs in the testa cells of the barley grain have been identified in six genes (Jende-Strid, 1993, 1995) and are being exploited by us to clone the genes encoding leucocyanidin reductase and a putative condensing enzyme by differential transcript display techniques. We have previously analyzed the accumulation of PAs and characterized dihydroxy-flavanol reductase activity and polymer formation in leaves of sainfoin (*Onobrychis viciifolia*) (Koupai-Abyazani et al., 1993a; Lees, Suttill, and Gruber, 1993; Lees, Gruber, and Suttill, 1995). In the present investigation we have studied the developmental pattern and cellular distribution of PA and anthocyanin in leaves, flowers, and seeds of alfalfa (*Medicago sativa*), *Lotus japonicus, Lotus uliginosus, Hedysarum sulfurescens,* and the woody legume *Robinia pseudacacia* and measured the activity of leucocyanidin reductase (LCR) in relation to PA formation. The results will enable the selection of suitable tissues and developmental stages for enzyme isolation.

MATERIALS AND METHODS

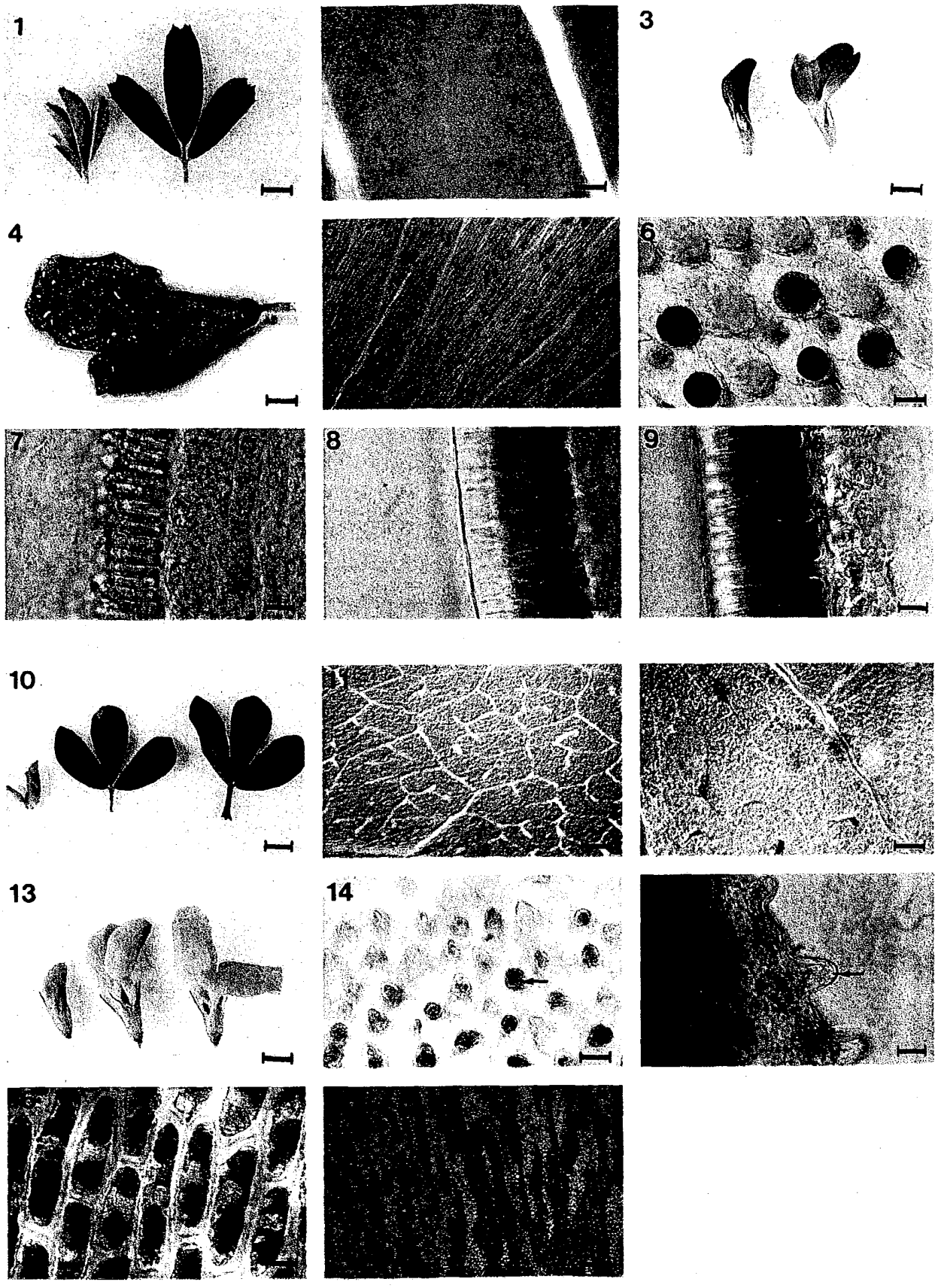
Plant material—Developing leaves and flowers from alfalfa (*Medicago sativa* L. *var.* Beaver), *Lotus japonicus* L., *Lotus uliginosus* L., *Hedysarum sulfurescens* L., and *Robinia pseudacacia* L. were harvested from 1- to 2-yr-old greenhouse plants. *R. pseudacacia* seeds were collected from a mature tree growing in a garden at the Carlsberg Laboratory. Seeds of other species were obtained from the legume seed collection at Agriculture Canada, Saskatoon. Developing alfalfa seeds were dissected from pods of manually cross-pollinated Beaver genotypes, which have medium to dark blue flowers. Fresh, rinsed tissue was sorted into stages of development on damp filter paper, blotted to remove excess water, then used fresh for cytology and chemical assays, or quick frozen in liquid N_2 and stored at −80°C for enzyme assays. Stained and fresh samples were photographed through a Zeiss Axioplan NMC100 Universal microscope. Developing seeds were frozen and cut into 15-μm thick sections on a freeze microtome.

Staining of proanthocyanidins—For method A, fresh tissue was incubated for 10 min in 1% vanillin in 6 mol/L HCl at room temperature, a procedure in which the terminal flavanol of PA polymers is labeled and a red color formed (Sakar and Howarth, 1976). Vanillin reacts strongly with both flavan-3-ol monomers and polymers, but weakly with dihydrochalcones and 4-deoxy-flavanols. For method B, whole tissue was washed for 2 d by soaking in several changes of MeOH until the MeOH was colorless, then soaked in butanol-HCl (70/30 volume/volume) at room temperature. The tissue was examined microscopically over a 1–5 d period, during which time PAs present in the tissue slowly hydrolyzed to orange-red or purple-red anthocyanin monomers. Hydrolysis was hastened by incubation at 80°C for up to 1.5 h, after an initial 2-h incubation at room temperature to fix the tissue and prevent disintegration.

Staining of anthocyanins—Method C involved staining as for method B, but a red color observed immediately upon addition of butanol-HCl indicated the presence of anthocyanins, since these compounds form the colored flavylium ion in an acidic environment. Method D used fresh tissue incubated in 50% HCl at room temperature, which resulted in development of a red color in the absence of a methanol prewash when anthocyanins were present.

Determination of PA content—Leaf and flower PA contents were measured by a modification of the butanol-HCl hydrolysis assay (Watterson and Butler, 1983). Fresh plant tissue (100 mg) was homogenized with a Polytron® at half speed for 1 min in 5 mL 100% MeOH with 200 mg polyvinylpolypyrrolidone (PVPP) to bind PAs, followed by centrifugation to form a pellet, which was extensively washed with MeOH to remove anthocyanins. Alfalfa developing seed tissue was ground fine first in liquid N_2 and then with 1 mL MeOH, mixed with 0.5 g PVPP/g seed tissue (mass/mass) seed tissue and handled as above. Bound polymer in the washed pellet was hydrolyzed for 1.5 h at 80°C with 7 mL butanol-HCl (70/30 volume/volume) and 80 μL 2% $NH_4Fe(SO_4)_2 \cdot 12H_2O$ in 2 mol/L HCl, releasing red-colored anthocyanidins into the solvent. The samples were clarified by centrifugation, and the height of the supernatant absorption peak at 550 nm was measured after correcting for baseline differences among the tissues. PA content of developing alfalfa seeds was calculated using a standard curve of polymer isolated from mature alfalfa seeds and expressed as a function of fresh mass. Polymer isolated from sainfoin (*Onobrychis viciifolia*) leaves was used to quantify PAs in other species.

2,3-trans-3,4-cis-leucocyanidin reductase (LCR) assays—LCR activity was measured according to Tanner and Kristiansen (1993). Liquid N_2-frozen leaves and flowers (500 mg) were ground to a dry fine powder, then reground in 3 mL LCR extraction buffer [100 mmol/L Tris-HCl, pH 8.0; 1 mmol/L EDTA, pH 8.0; glycerol (8.5% mass/volume); PEG 4000-8000 molecular mass (0.2% mass/volume); 5 mmol/L ascorbic acid; 5 mmol/L dithiothreitol (DTT) and 1 mmol/L phenylmethylsulfonylfluoride (PMSF)] to a fine porridge, followed by grinding with 200 mg PVPP. Samples (2 mL) were centrifuged twice for 10 min each at 10000 × *g* in a microfuge, and fresh supernatants were assayed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without further treatment or following desalting through Nick® columns. Frozen alfalfa seeds were processed in a similar fashion, except that fresh tissue mass varied between 35 and 370 mg. ^{3}H-*cis*-leucocyanidin substrate was prepared according to Tanner and Kristiansen (1993) and stored as aliquoted 10× stocks at −80°C. Assays (100 μL) contained 10 μL substrate (2 × 10^6 DPM), 10 μL NADPH (50 mmol/L) and tissue extract diluted to 80 μL in LCR assay buffer [100 mmol/L Tris-HCl, pH 7.5; 0.1 mmol/L EDTA; 1 mmol/L DTT and 8.5% glycerol (mass/volume), which had been sterilized by filtration and degassed by N_2 purge. Concentrated stocks of ^{3}H-substrate and NADPH were diluted with assay buffer, and either heat-denatured tissue extracts or desalted extracts without additional NADPH were used as control assays. Assays were incubated at 30°C for 30 min, then extracted three times with 300 μL H_2O-saturated ethyl acetate after addition of 50 μL unlabelled (+)-catechin (100 mmol/L). Pooled ethyl acetate fractions containing ^{3}H-flavanols were dried at room temperature under a N_2 stream, redissolved in 100 or 200 μL 0.22 μm-mesh filtered water, and separated isocratically by high performance liquid chromatography (HPLC) using 3% acetic acid on a Novapack C18 column (3.9 × 150 mm) (Waters) at a flow rate of 1 mL/min. ^{3}H-(+)-catechin was detected by a UV detector coupled to a Berthold flow-through scintillation detector (1 mL cell). Since *cis*-leucocyanidin slowly degraded on the HPLC column and left a slightly raised baseline, the radioactivity in the (+)-catechin peak was integrated after manual baseline construction using Berthold HPLC software, then expressed as a function of tissue fresh mass per hour of reaction time.

RESULTS

Alfalfa (Medicago sativa)—Figure 1 illustrates the stages of developing alfalfa leaves analyzed in this study. No red color was observed in leaves of any stage with any of the staining methods (Fig. 2), indicating that no PAs or anthocyanin are present in leaves of alfalfa. The stages of flower development analyzed are shown in Fig. 3. All flower stages immediately stained bright pink-red for anthocyanins with methods C and D (Fig. 4). The intensity of the pink stain correlated with the intensity of the purple-red color in fresh alfalfa flower petals and, depending on the genotype, it was either constant during flower development or declined in intensity after anthesis. This color was observed in all sections of the keel, side, and standard petals, except for some genotypes in which the standard petal base was white in mature flowers. In addition, cells with large dark blue or red vacuoles were interspersed among the pink-stained cells. These vacuolated cells were sometimes aligned in stripes radiating outward from the petal base, but were usually dispersed throughout the tissue and were especially dense at the petal tip (Fig. 5). Closer observation of a stained standard petal revealed that most petal cells also contained a number of very tiny red or blue vacuoles (data not shown). Large-colored vacuoles located in papilla on both flower surfaces could also be seen in fresh tissue (Fig. 6). The flower was uncolored using staining method B, indicating the absence of proanthocyanidins in the tissue.

Alfalfa seeds developed a brown-red color slowly with method B, but not with methods C and D, indicating that PAs rather than anthocyanins were present in this tissue. The color was first observed at the hilum of the seeds at 7–14 d after pollination (DAP), and by 28 d had completely covered the whole seed (data not shown). This was confirmed by staining frozen sections of developing seeds (7, 14, and 28 DAP) using method A. PAs were only located in the testa and stained light red by 14 DAP (Fig. 7). By 21 d seeds had a well-developed testa layer (Fig. 8), which changed little in structure by 36 DAP (Fig. 9), at which time the PA-containing cells stained dark red. Mature seed halves stained in a similar fashion also accumulated PAs only in the testa layer (data not shown).

In order to determine the content of PAs in developing alfalfa tissues, as well as to investigate whether they synthesize PAs or anthocyanin, homogenized tissue was mixed with PVPP, washed with MeOH and heated in butanol-HCl. Since PVPP initially binds both anthocyanins and PAs, control experiments were undertaken with *H. sulfurescens* flowers, which contain both of these compounds to determine the possibility of their separation. From homogenates of these tissues and of alfalfa flowers lacking PAs, anthocyanins could be selectively removed from the PVPP by extensive MeOH washes before hydrolysis to the point that they contributed very little to the final absorbance. It was found that in developing alfalfa seeds PA content rose to a maximum of 40 μg/mg fresh mass at 18 d postpollination, then declined to half maximum by 28 d (Fig. 18A). PA-PVPP binding could not be detected in developing alfalfa flowers or leaves at any stage.

LCR activity (Fig. 19A) was measured during tissue development by HPLC assays of (+)-catechin, from 3,4-*cis*-leucocyanidin (Fig. 19B). While NADPH-dependent LCR activity could not be detected in developing flowers or leaves, there was considerable activity in seeds at 14 DAP (Fig. 18A). It declined rapidly and reached a 35-fold lower value by 21 DAP.

Lotus japonicus—Figure 10 illustrates the stages of developing *L. japonicus* leaves investigated. Leaf cells

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Figs. 1–17. *Medicago sativa*. **1.** (Fresh) unopened and mature leaves. Bar = 5 mm. **2.** Proanthocyanidins (PA) are absent in butanol-HCl, heat treated mature leaves (bar = 10 μm). **3.** (Fresh) unopened and mature flowers. Bar = 3.5 mm. **4.** Anthocyanins stain red upon 50% HCl treatment of mature flowers. Bar = 1.2 mm. **5.** Standard petal treated with butanol-HCl and heat (method C) showing rapidly staining anthocyanins. Bar = 5 μm. **6.** (Unstained) papillae cells from standard petal. Bar = 1 μm. **7.** Cross section of a seed 14 d after pollination (DAP) stained with 1% vanillin-HCl. Bar = 2.5 μm. **8.** Cross section of seed 21 DAP stained with 1% vanillin-HCl. Bar = 2.5 μm. **9.** Cross section of seed 36 DAP stained with 1% vanillin-HCl. Bar = 2.5 μm. *Lotus japonicus*. **10.** Leaves, stage I: folded in bud (left), stage II: first completely open trifoliate close to bud (middle), stage III: trifoliate half-way down the stem (right). Bar = 5 mm. **11.** Proanthocyanidins (PA) are absent in butanol-HCl, heat-treated mature leaves. Bar = 10 μm. **12.** A light orange stain appearing upon prolonged butanol-HCl hydrolysis (5 d) of leaves from plants grown under "crowded" conditions. Bar = 10 μm. **13.** Flowers, stage I: green, unopened (left), stage II: yellow-green, partially opened (middle), stage III: fully opened standard petal but closed wing petals, bright yellow (right). Bar = 4 mm. **14.** Standard petal treated with butanol-HCl showing PA stain located in papilla (arrow) from stage III flowers. Bar = 2.5 μm. **15.** Fresh stage III standard petal showing clear uncolored papilla (arrow) on surface cells. Bar = 2.5 μm. **16.** As for Fig. 14 but with flattened papillae. Bar = 1 μm. **17.** A patchy network appears in stage III standard petals upon butanol-HCl treatment without heat. Bar = 5 μm.

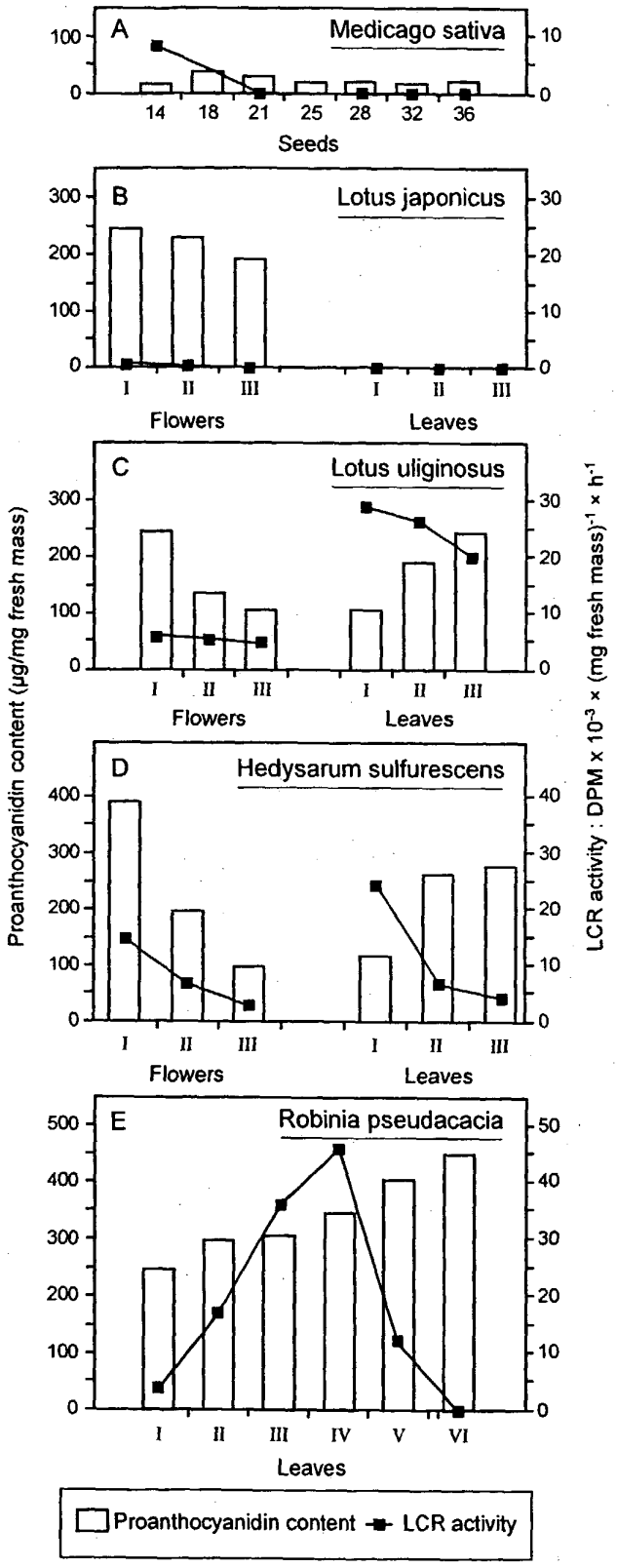


Fig. 18. Proanthocyanidin (PA) content and leucocyanidin reductase (LCR) activity in legume species during tissue development of seeds, leaves, and flowers. (A) *Medicago sativa* seeds. (B) *Lotus japonicus* flowers and leaves. (C) *Lotus uliginosus* flowers and leaves. (D) *Hedysarum sulfurescens* flowers and leaves. (E) *Robinia pseudacacia* leaves. Standard deviation within 10% of each data point, with the exception of alfalfa seed in which it was within 20%. Average of 3–5 experiments.

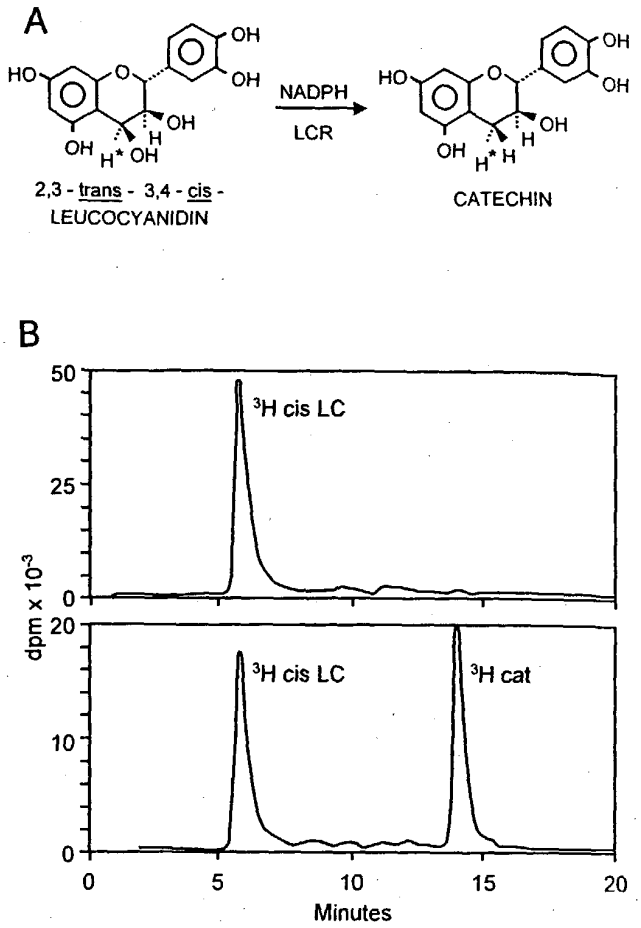


Fig. 19. Enzymatic reduction of ^{3}H(+)-2,3-*trans*-3,4-*cis*-leucocyanidin (LC) to ^{3}H(+)-catechin. (A) Chemistry of the reaction, * indicates the labeled hydrogen atom. (B) HPLC traces of precursor and product from an LCR activity assay of 14-d-old alfalfa seeds. Top panel: *cis*-LC elutes at a retention time (RT) of 6 min (standard). Bottom panel: in the presence of LCR, (+)-catechin (RT 14 min) is formed.

did not stain red with any of the staining methods at any stage of development (Fig. 11). Occasionally, long red cells were observed in the midvein of leaves (data not shown). When *L. japonicus* plants were grown under "crowded" conditions, a light orange stain on veins and vein ends of stage II and III leaves was present after a prolonged butanol-HCl hydrolysis (4–5 d) (Fig. 12).

L. japonicus develops single or double yellow flowers, arising from separate bracts. The three stages of flower development studied are depicted in Fig. 13. Young and mature wing and keel petals stained yellow-brown to a similar extent across all portions of the tissue upon slow butanol-HCl hydrolysis, then dark red upon further heating. Dark orange containing veins were present throughout

these stained floral organs. Pollen, pistil, anthers, developing seed pod, and calyx tissue also stained completely dark red under these conditions. Stained standard tissue was structurally similar at different stages of development, with orange-brown stained material developing slowly in clear, uncolored erect papillae stretching outward from the surface of the petal (Figs. 14, 15), followed by a dark red color after heating. The surface cells of fresh flower tissue also contained small yellow vacuoles (arrow) located toward the inside of the petal and distinct from the yellow-stained material in the papillae. When the papillae were flattened during microscopic examination, the orange-brown stain filled more than half the cell (Fig. 16). As the flower matured between stage II and III (Fig. 13), a patchy red-stained PA network developed from the base to the middle of the standard petal (Fig. 17), but excluding the tip. Since it was difficult to focus on this network from either surface, it is apparently sandwiched between the two cell layers with the papillae. Anthocyanins were only observed at the base of fresh standard tissue in cherry red cells identified by acidic treatment (data not shown). The intensity of the stained anthocyanin stripes increased as the flower opened completely.

When frozen sections of developing and mature seeds of *L. japonicus* were stained according to method A, the dark red color was limited to the testa layer, confirming that PAs accumulate only in this tissue (data not shown).

PA content in immature flowers of *L. japonicus* amounted to ≈250 μg/mg fresh mass (Fig. 18B) and declined slightly in fully mature flowers. PA was not detectable in any extracts of *L. japonicus* leaves (Fig. 18B). Developing flowers had very low LCR activity, which disappeared during flower maturation, while LCR activity could not be measured in *L. japonicus* leaves at any stage of development (Fig. 18B).

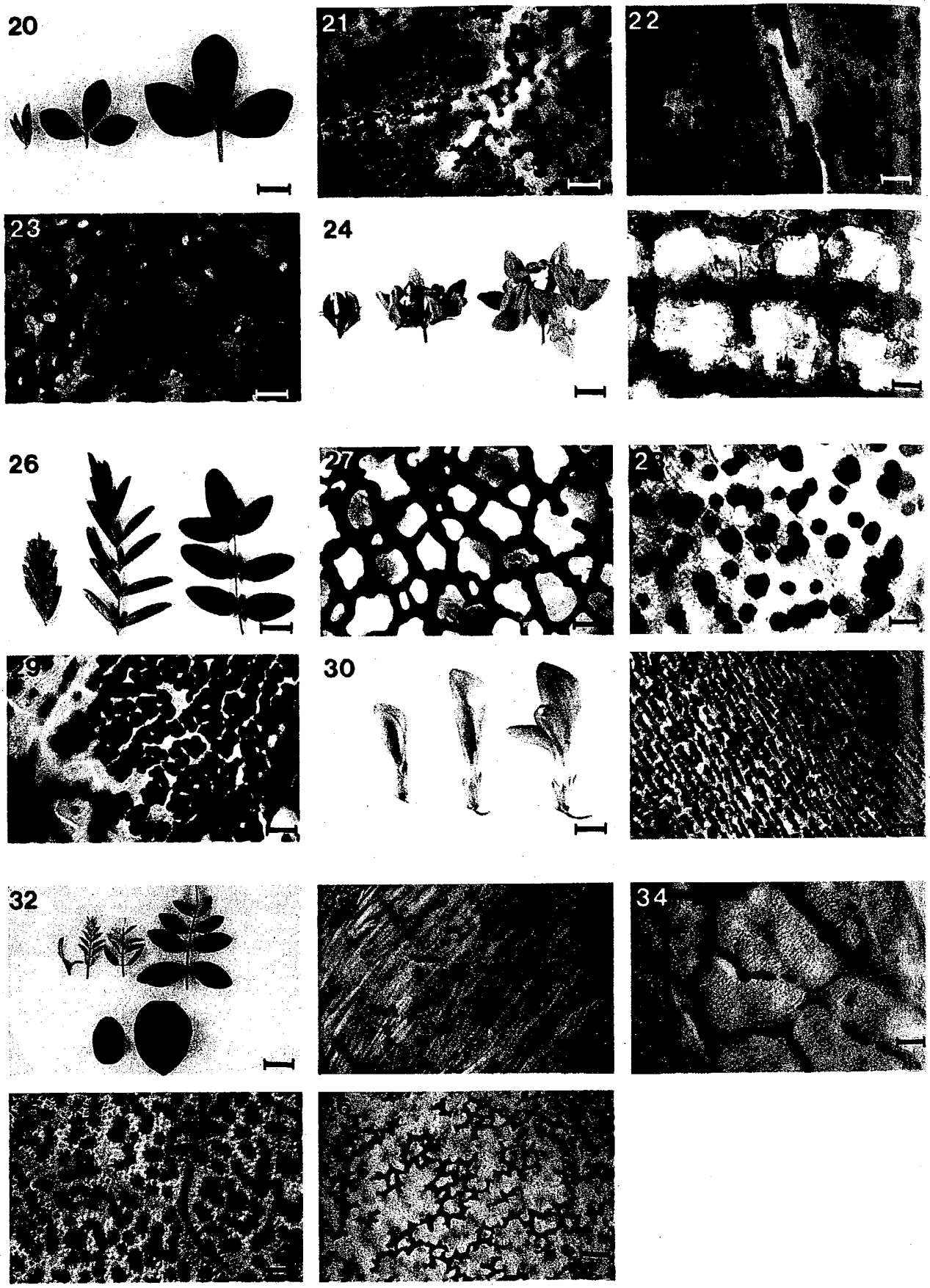
Lotus uliginosus (L. pedunculatus syn.)—Figure 20 illustrates stages in the development of *L. uliginosus* leaves. These are larger and more hairy than *L. japonicus* leaves, and possess large numbers of small subepidermal mesophyll-like cells on the adaxial leaf surface, which stained dark orange at a relatively early stage of leaf development using method C (Fig. 21). These cells, as well as long cells above the leaf veins, also stained with method A (Fig. 22). In addition to the smaller stained cells, new large, more heavily stained mesophyll-like cells appeared first on the adaxial surface of the leaf edge at stage III (data not shown). *L. uliginosus* leaves also first developed a discontinuous subepidermal network of stained cells on the abaxial surface, beginning at stage II and continuing into full maturity (Fig. 23). Leaf tissue did not stain red immediately upon addition of the hydrolytic solvent or acid alone at any stage of development, indicating that no anthocyanins were present.

L. uliginosus flowers are produced as a composite yellow flower head with 4–6 florets subtended by a single bract (Fig. 24). The fresh flowers have thin red stripes radiating outward from the standard petal base toward the edge on the exposed inner surface. *L. uliginosus* flowers stained bright orange-red using methods A and B, but with a structure distinct from that of *L. japonicus* as follows. An unstructured network was observed at flower stage I (data not shown), while stages II and III looked similar after staining and contained a three-dimensional well-connected thick rectangular network of orange cells (Fig. 25). This network covered most of the exposed portion of the standard petal, beginning at the fold, not including the base, and becoming patchy at the side and tip. This is distinct from the thin, patchy planar network observed in *L. japonicus* standard petals, but similar in that it also appeared to be sandwiched between the papillae. Unlike *L. japonicus, L. uliginosus* papillae did not stain using any method, but the sexual parts, pods, and calyx stained dark red.

Frozen sections of developing and mature seeds of *L. uliginosus* were stained by method A, and a dark red color showed that PAs accumulate only in the testa layer. PA content increased during tissue development to a maximum of 250 μg/mg fresh mass in mature *L. uliginosus* leaves (Fig. 18C). In contrast, PA content in developing flowers declined from 250 μg/mg fresh mass to one-half of this value. The LCR activity of *L. uliginosus* flowers remained constant at 5 000 DPM·mg fresh mass^{-1}·h^{-1} during development. The maximum LCR activity in leaves was sixfold higher than in flowers, and the activity declined by 30% during leaf development (Fig. 18C).

Hedysarum sulfurescens—Young *H. sulfurescens* leaves (Fig. 26) were structurally similar to mature leaves when observed fresh or stained with method A or B. Stages II and III had a well-developed continuous subepidermal planar network on the abaxial leaf surface, which stained red with either method A or B (Fig. 27), as well as large numbers of red-stained cells in the mesophyll layer (Fig. 28). These cells were large, clear, and colorless in fresh tissue. The network was structurally similar in all stages, except at the base of the leaf where the stage III network was compressed (Fig. 29). Stage III mesophyll PA cells appeared to leak stained material more readily into the solvent. Long red cells appeared over the main leaf vein after staining with method B (data not shown). Leaves turned red when stained by method D (data not shown), indicating the presence of anthocyanins in this tissue. However, the color did not leak into the medium and could not be completely removed by MeOH soaking. Closer observation of these leaves showed small dark-stained vacuoles similar to those in alfalfa flowers, as well as brown patches, which only occurred in stage III leaves (data not shown). These vacuoles and patches may have contributed to the brown-green color observable in fresh greenhouse leaf material.

H. sulfurescens stage I flowers, which were normally pink-orange colored when fresh (Fig. 30), developed a bright pink color only at the flower tip when stained for anthocyanins by method D (data not shown). Stage II flowers stained bright pink over the tip and base of the keel petal, over all parts of the wing petals, and on both sides of the standard petal between long veins, but not on the middle, tip, or base of the standard (data not shown). Nonpetal floral organs did not stain with acid. Old flowers (stage III) had very little color after staining and were mostly yellow from the color of the anthers. If the flower had been previously soaked in several changes of MeOH prior to staining, only a faint pink color developed, indicating that most of the anthocyanins could be removed.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After staining *H. sulfurescens* flowers by method B, a dark red color occurred, indicating the presence of PAs. All organs including all petals, anthers, stamens, pistil, calyx, and seed pod, were stained, with the color most concentrated in the seedpod and calyx. The keel petal stained intensely on the edges and tip, while the wing petals stained lightly all over. The standard petal stained intensely in the middle section (Fig. 31) and lightly on the tip and side edges. Staining was especially intense close to long veins. Stage I flowers appeared similar in organization to the mature stages after staining with this method.

When frozen sections of developing and mature seeds of *H. sulfurescens* were stained by method A, a dark red color showed that PAs accumulate only in the testa layer.

PA content measured by PVPP binding doubled during maturation of *H. sulfurescens* leaves to the level measured in *L. uliginosus* leaves, while the LCR activity declined from 24000 to 7000 DPM·mg $FW^{-1}\cdot h^{-1}$ (Fig. 18D). This is in contrast to the situation in developing *H. sulfurescens* flowers in which the decline in PVPP-bound material is correlated with a decline in LCR activity (Fig. 18).

Robinia pseudacacia—Figure 32 illustrates the stages of *R. pseudacacia* developing leaves used in this study. Leaves stained orange-red with method B at very early stages of development, i.e., stages I–III (Fig. 33). As the leaf matured and began to open, the tissue became more organized. Longer red-stained cells were observed layered over a multitude of small, interconnected veins throughout the leaf (Fig. 34), but red-stained cells were not observed over the main leaf vein. These structures developed during stages II–IV. Between stages III and V, large numbers of orange-red to purple-stained cells developed in the mesophyll layers on the adaxial side of *R. pseudacacia* leaves stained with either method A or B (Fig. 35) and by stage V an orange-stained discontinuous network had formed over part of the abaxial leaf surface (Fig. 36). Many of the mesophyll PA cells had burst open by stage VI, and the network was no longer visible at this stage. *R. pseudacacia* leaves were also stained with acid to detect the presence of anthocyanins. The main leaf vein stained light pink immediately with method C or D, but no red color developed in the rest of the tissue at any stage of development. Acid-stained leaves had tiny brown flecks where viewed across the leaf surface. This staining reaction is not characteristic of anthocyanins.

Fresh *R. pseudacacia* flowers were light green before petal opening and white with green veins at full maturity. Floral organs did not stain with methods A, C, or D at any stage of development.

When frozen sections of developing and mature seeds of *R. pseudacacia* were stained by method A, a dark red color showed that PAs accumulate only in the testa layer. Developing *R. pseudacacia* leaves contained the largest amount of PA of all the legumes tested, and the content continued to rise with leaf expansion until stage VI (Fig. 18E). A small portion of the color measured in the PVPP-binding assay may be due to anthocyanins, since exhaustive extraction of the leaf with MeOH did not eliminate staining with acid completely. The LCR activity also rose during leaf development until stage IV, after which time the activity declined dramatically to undetectable levels (Fig. 18E). PA and LCR activity could not be measured in *R. pseudacacia* flowers at any stage of development.

DISCUSSION

The accumulation of anthocyanins and proanthocyanidins (PA) was investigated in a variety of legume species during tissue development in the seed, leaf, and flower. Table I summarizes the chemical data for proanthocyanidins and the presence of leucocyanidin reductase (LCR) activity. Developing legume seeds synthesize and deposit PA in the testa cells without concomitant synthesis of anthocyanins, unlike barley seeds, which synthesize both compounds (Jende-Strid, 1993). Cytological analysis showed that anthocyanins and PAs did not appear to be synthesized within the same cell types in any of the tissues except in *H. sulfurescens* flower tissues. PAs and LCR could not be detected in alfalfa flowers or leaves, *R. pseudacacia* flowers, nor in *L. japonicus* leaves. The lack of PA and LCR in alfalfa leaves and flowers supports previous studies in which large numbers of different alfalfa varieties were screened, and no genotypes were

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Figs. 20–36. Tests for proanthocyanidins in leaves and flowers of *Lotus uliginosus, Hedysarum sulfurescens,* and *Robinia pseudacacia. Lotus uliginosus.* **20.** Leaves, stage I: folded in bud (left), stage II: first completely open trifoliate close to bud (middle), stage III: trifoliate half way down the stem (right). Bar = 5 mm. **21.** Stage I leaf tissue, adaxial surface proanthocyanidins stain red upon butanol-HCl treatment (without heat). Bar = 2.5 μm. **22.** Staining with 1% vanillin-HCl of proanthocyanidin (PA) viewed from the adaxial surface of stage II leaf tissue. Bar = 2.5 μm. **23.** PA staining in the abaxial surface of stage III tissue revealing a network of PA-containing cells. Bar = 2.5 μm. **24.** Flowers, stage I: green, unopened (left), stage II: yellow-green, partially opened (middle), stage III: fully opened standard petal but closed wing petals, bright yellow (right). Bar = 6 mm. **25.** Butanol-HCl, heat-treated standard petal (stage II) displaying an orange three-dimensional PA-containing network. Bar = 1 μm. *Hedysarum sulfurescens.* **26.** Leaves, stage I: unfolded leaflets 0.2–1.5 cm long close to bud (left), stage II: fully open leaflets from middle of stem (middle), stage III: fully open, dark green, mature leaflet (right). Bar = 9.5 mm. **27.** Stage II leaves (abaxial surface) containing a continuous red proanthocyanidin (PA) network is seen upon staining with butanol-HCl and heating. Bar = 1 μm. **28.** Mesophyll-like cells in stage II leaves (abaxial surface) staining red upon butanol-HCl and heat treatment. Bar = 2.5 μm. **29.** Same as 27, but stage III leaf and showing a compressed network. Bar = 1 μm. **30.** Flowers, stage I: unopened (left), stage II: half open (middle), stage III: mature (right). Bar = 2 mm. **31.** PA stained by butanol-HCl and heat in the middle section of a standard petal at stage II. Bar = 5 μm. *Robinia pseudacacia.* **32.** Leaves (stages left to right, top to bottom), stage I: brown-green buds <2 mm wide (left), stage II: distinct folded brown-green leaflets <0.5 cm long, stage III: folded leaflets 0.5–2.0 cm long, stage IV: shoot with half opened brown-green leaflets; bottom, stage V: completely open lighter green leaflets, stage VI: completely open dark-green leaflets from older part of stem. Bar = 18 mm. **33.** Proanthocyanidins stained red by butanol-HCl (with heat) in a leaf at stage I. Bar = 2.5 μm. **34.** Butanol-HCl and heat treatment of a leaf at stage IV revealing proanthocyanidins (PA) in cells overlying leaf veins. Bar = 2.5 μm. **35.** Same conditions as in Fig. 34, but adaxial leaf surface. Bar = 5 μm. **36.** At stage V, PA stained red by butanol-HCl and heat revealing a discontinuous network (abaxial surface). Bar = 5 μm.

TABLE 1. Proanthocyanidin (PA) content, leucocyanidin reductase (LCR) activity, and location of PAs in aerial parts of legumes. ND = not determined.

Plant species	Tissue	Anthocyanins	PA	LCR activity	Location of PA
Medicago sativa	seed	−	+	+	testa
	leaf	−	−	−	
	flower	+	−	−	
Lotus japonicus	seed	−	+	−	testa
	leaf	−	−	−	
	flower	+	+	+	parenchyma, sexual parts, pod calyx
Lotus uliginosus (*L. pedunculatus* syn.)	seed	−	+	ND	testa
	leaf	+	+	+	mesophyll/subepidermal network
	flower	+	+	+	parenchymal network, sexual parts, pod, calyx
Hedysarum sulfurescens	seed	−	+	ND	testa
	leaf	+	+	+	mesophyll/subepidermal network
	flower	+	+	+	parenchyma, sexual parts, pod, calyx
Robinia pseudacacia	seed	−	+	ND	testa
	leaf	+	+	+	mesophyll/subepidermal network/veins
	flower	+	−	−	

found expressing PAs in the leaves or flowers (Goplen et al., 1980; Marshall et al., 1981).

The PA content of alfalfa seeds rose during development and then declined slightly with increasing seed mass (Fig. 18A). This reduction is likely due to the early cessation in LCR activity, which was observed after the maximum PA content was reached and when the testa layer appeared completely filled. The importance of an LCR enzyme that uses the dihydroxyflavanol substrate, 2,3-*trans*-3,4-*cis*-leucocyanidin, as a key step in alfalfa seed PA synthesis, is supported by a study in which the alfalfa seed PA polymer was found to be composed of mainly the dihydroxy subunit procyanidin (Koupai-Abyazani et al., 1993b). While the PAs in developing seeds of the other legume species were all located in testa cells, LCR was not measured due to difficulty in isolating sufficient plant material for enzyme assays.

PA content declined during development in all flowers accumulating these compounds. LCR activity also declined during *L. japonicus* and *H. sulfurescens* flower development, but remained constant in *L. uliginosus* flowers. This suggests that there may be degradation of the polymers during flower maturation, a situation similar to that of sainfoin leaf PA cells, which appear to empty their PA content during late maturity and senescence (Lees, Suttill, and Gruber, 1993). However, the high LCR activity in *L. uliginosus* flowers indicates that PA synthesis is still proceeding in this plant, but at a lower rate than degradation.

The high initial content of PA together with the low initial LCR activity in *L. japonicus* flowers could result from the presence of a second flavanol reductase enzyme, which would require different cofactors and substrate and which might be active at a different stage of flower development. This could also explain the rise in PA content and concurrent decline in LCR activity during maturation of *L. uliginosus, H. sulfurescens,* and *R. pseudacacia* leaves. This hypothesis is supported by the fact that mature leaf PA isolated from all three of these species is mainly composed of trihydroxy subunits, which are derived from (+)-gallocatechin, (−)-epigallocatechin, and *cis*-leucodelphinidin (A. Muir, personal communication, Agriculture Canada, Saskatoon). Parallel investigations on *Onobrychis viciifolia,* a plant related to *H. sulfurescens* with two similar unique types of leaf PA cell structure, revealed that the PA composition changes toward a greater proportion of trihydroxy subunits during leaf development (Koupai-Abyazani et al., 1993a). In addition, PAs were shown to accumulate differentially in each of these cell types during leaf development (Lees, Suttill, and Gruber, 1993). LCR and a trihydroxyflavanol reductase activity also peak differentially during leaf development (B. Skadhauge, unpublished data).

It remains unclear whether different PA polymers and different reductases are uniquely compartmentalized in the specialized PA cell types noted in *O. viciifolia, H. sulfurescens,* and *R. pseudacacia* leaflets. This would be relatively easy to determine, since the network layer of PA cells can be stripped off with the abaxial epidermis in both mature *O. viciifolia* and *H. sulfurescens* leaves. The differences in PA content and LCR activity between *L. uliginosus* flower and leaf tissues may also reflect the appearance of new large mesophyll cells and a PA network at late stages of leaf maturation, whereas the structure of the flower PA cells remained more constant during development.

L. uliginosus leaf extracts had the highest continuous LCR activity during tissue development of all the legumes tested. *H. sulfurescens* leaves and flowers, *L. uliginosus* flowers, *R. pseudacacia* leaves and alfalfa seeds only contained active enzyme at young stages of development. As sources of plant material for enzyme isolation, the following disadvantages should be mentioned: *H. sulfurescens* produces only a small number of leaves and flowers at any time, *R. pseudacacia* is a tree legume with progressively thickening leaves, making it difficult to harvest enough developing tissue, and young alfalfa seeds are difficult to dissect. Since *L. uliginosus* is a perennial forage legume and large amounts of fresh leaves can be harvested routinely from greenhouse or field-grown material, it may be an ideal plant from which to purify LCR to homogeneity.

PAs and LCR activity could not be detected in *L. japonicus* leaves, although large amounts of PA accumulated in the flowers. *L. japonicus* is a diploid, self-pollinating plant, which is transformable using *Agrobacterium*

tumefaciens (Handberg and Stougård, 1992), and a transposon tagging system has recently been described for this plant (Thykjær et al., 1995). These features, including some rapid PA-screening techniques and the fact that many of the *Lotus* species do synthesize leaf PAs, make *L. japonicus* a suitable plant species for the isolation of transposon mutants. With such aid, it should be feasible to isolate DNA sequences encoding legume structural and regulatory genes for PA biosynthesis.

LITERATURE CITED

CHAN, B. G., A. C. WAISS, AND M. LUKEFAHR. 1978. Condensed tannin and antibiotic chemical from *Gossypium hirsutum*. *Journal of Insect Physiology* 24: 113–118.

DAKORA, F. D. 1995. Plant flavonoids: biological molecules for useful exploitation. *Australian Journal of Plant Physiology* 22: 87–99.

DEBOO, G. B., M. C. ALBERTSEN, AND L. P. TAYLOR. 1995. Flavanone 3-hydroxylase transcripts and flavonol accumulation are temporally coordinated in maize anthers. *Plant Journal for Cell and Molecular Biology* 7: 703–713.

DIXON, R. A., AND N. L. PAIVA. 1995. Stress induced phenylpropanoid metabolism. *Plant Cell* 7: 1085–1097.

DOONER, H. K., T. P. ROBBINS, AND R. A. JØRGENSEN. 1991. Genetic and developmental control of anthocyanin biosynthesis. *Annual Review of Genetics* 25: 173–199.

ERDAL, K. 1986. Proanthocyanidin-free barley-malting and brewing. *Journal of the Institute of Brewing* 92: 220–224.

GOPLEN, B. P., R. E. HOWARTH, S. K. SAKAR, AND K. LESINS. 1980. A search for condensed tannins in annuals and perennial species of *Medicago, Trigonalla* and *Onobrychis*. *Crop Science* 20: 801–804.

HANDBERG, K., AND J. STOUGÅRD. 1992. *Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics. *Plant Journal* 2: 487–496.

HARA, Y. 1994. Antioxidant action of tea polyphenols. *International Biotechnology Laboratory* (December), 14–15.

HARBORNE, J. B. 1988. The flavonoids: advances in research since 1980. Chapman and Hall, New York, NY.

HARTWIG, U. A., C. M. JOSEPH, AND D. A. PHILLIPS. 1991. Flavonoids released naturally from alfalfa seeds enhance growth rate of *Rhizobium meliloti*. *Plant Physiology* 95: 797–803.

HEDIN, P. A., AND S. K. WAAGE. 1986. Roles of flavonoids in plant resistance to insects. Plant flavonoids. *In* Biology and medicine biochemical, pharmacological and structure activity relationships, 87–100. Alan R. Liss, New York, NY.

HOLTON, T. A., AND E. C. CORNISH. 1995. Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell* 7: 1071–1083.

HUNGRIA, M., C. M. JOSEPH, AND D. A. PHILLIPS. 1991. Anthocyanins and flavonols, major *nod*-gene inducers from seeds of a black-seeded common bean (*Phaseolus vulgaris* L.). *Plant Physiology* 97: 751–758.

JENDE-STRID, B. 1993. Genetic control of flavonoid biosynthesis in barley. *Hereditas* 119: 187–204.

———. 1995. Coordinator's report: anthocyanin genes. *Barley Genetic Newsletter* 24: 162–165.

JONES, W. T., J. W. BROADHURST, AND J. W. LYTTLETON. 1976. The condensed tannins of pasture legume species. *Phytochemistry* 15: 1407–1409.

KOUPAI-ABYAZANI, M. R., J. MCCALLUM, AND B. A. BOHM. 1992. Identification of the constituent flavonoid units in sainfoin proanthocyanidins by reverse-phase high-performance liquid chromatography. *Journal of Chromatography* 594: 117–123.

———, ———, A. MUIR, B. A. BOHM, G. H. N. TOWERS, AND M. Y. GRUBER. 1993a. Developmental changes in the composition of proanthocyanidins from leaves of sainfoin (*Onobrychis viciifolia* scrop.). *Journal of Agricultural and Food Chemistry* 41: 1066–1070.

———, ———, ———, G. L. LEES, B. A. BOHM, G. H. N. TOWERS, AND M. Y. GRUBER. 1993b. Purification and characterization of a proanthocyanidin polymer from seed of alfalfa (*Medicago sativa* cv. Beaver). *Journal of Agricultural and Food Chemistry* 41: 565–569.

KRISTIANSEN, K. N. 1984. Biosynthesis of proanthocyanidins in barley: genetic control of the conversion of dihydroquercetin to catechin and procyanidins. *Carlsberg Research Communications* 49: 503–524.

———. 1986. Conversion of (+)-dihydroquercetin to (+)-2,3-*trans*-3,4-*cis*-leucocyanidin and (+)-catechin with an enzyme extract from maturing grains of barley. *Carlsberg Research Communications* 51: 51–61.

———, AND W. ROHDE. 1991. Structure of the *Hordeum vulgare* gene encoding dihydroflavanol-4-reductase and molecular analysis of *ant*18 mutants blocked in flavonoid synthesis. *Molecular and General Genetics* 230: 49–59.

LEES, G. L., M. Y. GRUBER, AND N. H. SUTTILL. 1995. Condensed tannins in sainfoin. 2. Occurrence and changes during leaf development. *Canadian Journal of Botany* 73: 1540–1547.

———, N. H. SUTTILL, AND M. Y. GRUBER. 1993. Condensed tannins in sainfoin. 1. A histological and cytological survey of plant tissues. *Canadian Journal of Botany* 71: 1147–1152.

MARSHALL, D. R., P. BROVE, J. GRACE, AND J. MUNDAY. 1981. Tannin in pasture legumes 2. The annual and perennial *Medicago* species. *Australian Journal of Experimental Animal Husbandry* 21: 55–58.

MAXWELL, C. A., U. A. HARTWIG, C. M. JOSEPH, AND D. A. PHILLIPS. 1989. A chalcone and two related flavonoids released from alfalfa roots induce *nod* genes of *Rhizobium meliloti*. *Plant Physiology* 91: 842–847.

MCNABB, W. C., G. C. WAGHORN, T. N. BARRY, AND I. D. SHELTON. 1993. Effect of condensed tannins in *Lotus pedunculatus* on the digestion and metabolism of methionine, cystine and inorganic sulfur in sheep. *British Journal of Nutrition* 70: 647–661.

MELDGAARD, M. 1992. Expression of chalcone synthase, dihydroflavonol reductase and flavanone-3-hydroxylase in mutants of barley deficient in anthocyanin and proanthocyanidin biosynthesis. *Theoretical and Applied Genetics* 83: 695–706.

PETERS, N. K., J. W. FROST, AND S. R. LONG. 1986. A plant flavone luteolin induces expression of *Rhizobium meliloti* nodulation genes. *Science* 233: 977–980.

ROBBINS, M. P., T. R. CARRON, AND P. MORRIS. 1992. Transgenic *Lotus corniculatus*: a model system for modification and genetic manipulation of condensed tannin biosynthesis. *In* R. W. Hemmingway and P. E. Laks [eds.], Plant polyphenols, 111–133. Plenum Press, New York, NY.

SAKAR, S. K., AND R. E. HOWARTH.1976. Specificity of vanillin test for flavonols. *Agricultural and Food Chemistry* 24: 317–320.

SPENCER, D., T. J. V. HIGGINS, M. FREER, H. DOVE, AND J. B. COOMBE. 1988. Monitoring the fate of dietary proteins in rumen fluid using gel electrophoresis. *British Journal of Nutrition* 60: 241–247.

STAFFORD, H. A. 1990. Flavonoid metabolism. CRC Press, Boca Raton, FL.

TANNER, G. J., AND K. N. KRISTIANSEN. 1993. Synthesis of ^{3}H-3,4-*cis*-leucocyanidin and enzymatic reduction to catechin. *Analytical Biochemistry* 209: 274–277.

———, A. E. MOORE, AND P. J. LARKIN. 1994. Proanthocyanidins inhibit hydrolysis of leaf proteins by rumen microflora *in vitro*. *British Journal of Nutrition* 71: 947–958.

THYKJÆR, T., J. STILLER, K. HANDBERG, J. JONES, AND J. STOUGÅRD. 1995. The maize transposable element *Ac* is mobile in the legume *Lotus japonicus*. *Plant Molecular Biology* 27: 981–993.

YLSTRA, B., J. BUSSCHER, J. FRANKEN, P. C. H. HOLLMAN, J. N. M. MOL, AND A. J. VAN TUNEN. 1994. Flavonols and fertilization in *Petunia hybrida*: localization and mode of action during pollen tube growth. *Plant Journal* 6: 201–212.

WATTERSON, J. J., AND L. G. BUTLER. 1983. Occurrence of an unusual leucoanthocyanidin and absence of proanthocyanidins in sorghum leaves. *Journal of Agricultural Food Chemistry* 31: 41–45.

WETTSTEIN, D. VON., B. JENDE-STRID, B. AHRENST-LARSEN, AND J. A. SØRENSEN. 1977. Biochemical mutant in barley renders chemical stabilization of beer superfluous. *Carlsberg Research Communications* 42: 341–351.

CONDENSED TANNINS IN SOME FORAGE LEGUMES: THEIR ROLE IN THE PREVENTION OF RUMINANT PASTURE BLOAT

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ABSTRACT

For the past 20 years, the focus in our laboratory has been on finding the causes of ruminant pasture bloat and eventually breeding a bloat-safe alfalfa (*Medicago sativa* L.); i.e., with bloat potential reduced to the economic threshold. In the mid-seventies, the mechanisms of bloat were explored and found to be more physical than chemical. Characteristic of all bloating legumes after ingestion was a very rapid initial rate of ingestion by rumen microbes. Through the study of bloating and non-bloating legumes, factors were elucidated in the plant that would slow this process. One of these factors was the presence of condensed tannins in the herbage. Some of the non-bloating legumes contained these secondary metabolites, but no condensed tannins were found in any of the bloating legumes. Therefore, species containing an appreciable amount of condensed tannins in their leaves and stems are considered to be non-bloating. Conventional breeding methods have not been successful in producing an alfalfa with condensed tannins in its herbage. New approaches using tissue culture techniques are being attempted, but genetic engineering has the greatest potential for success.

INTRODUCTION

Pasture bloat is a condition that may occur when cattle feed on certain leguminous plants, or even some graminaceous, crops at the right stage of development. Bloat can commence quickly and is manifested in the inability of the animal to

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expel gases produced in the rumen from normal fermentation. Bloat is not a recent phenomenon, having been described in agricultural writings since at least A.D. 60[1] and known as a disorder in cattle for centuries.[2] The incidence of bloat has increased, however, with the introduction of forage legumes such as alfalfa and clover into cultivated pastures.[3] In the history of bloat, such names as hoove, hoven, tympany, and blown have appeared in English journals of the 18th and 19th centuries, to describe the disorder, and the French term 'météorisme', meaning the process of ballooning, is still used.[1] In the past, a number of causes for a 'bellyful of gas' have been cited including blockages caused by high consumption of dense feed, poisons, and excessive gas production. Experimental research from the 1940's to the 1960's was based on these and other explanations, and it was only after much debate that the frothy or foamy basis of pasture bloat was generally accepted over many other theories.[1] This acceptance resulted in an extensive research effort that further discarded the involvement of intracellular saponins and soluble proteins[4] as being directly responsible for the onset of bloat. As well, it became an accepted fact that the presence of tannins (for the purpose of this paper, tannin refers to condensed tannin) in the herbage of a forage was a bloat-safe character.[5] Alfalfa (*Medicago sativa* L.) is a highly nutritious, well-adapted legume that has been called the 'queen' of the forage crops. Its one major drawback is that it may induce bloat in cattle through very rapid tissue and cell breakdown during the early stages of digestion. Legumes such as sainfoin (*Onobrychis viciifolia* Scop.) and birdsfoot trefoil (*Lotus corniculatus* L.), which contain tannin in their herbage, are known to be non-bloating. No tannins have been found in alfalfa herbage although tannins are present in the seed coat. This paper will provide a brief description of ruminant pasture bloat, examine the tannins found in some legumes, discuss their relationship to the bloat-safe character, and describe attempts to increase the tannin content in alfalfa herbage through tissue culture.

PASTURE BLOAT

Pasture bloat has been described as an insidious disease.[6] It remains a continuing hazard to milk producers and cattlemen because of its unpredictability and speed of onset.[7] Bloat can occur suddenly in pastures where there was no previous incidence of the condition, resulting in significant animal losses to individual farmers or ranchers (fig. 1). The problem is worldwide and creates a real deterrent for using highly nutritious legumes such as alfalfa and clover in pasture. Financial losses due to bloat in the beef cattle and dairy industry are substantial. In the United States and Canada, losses due to cattle deaths account for approximately 1 percent of the animals grazing legume pastures[8] with yearly monetary losses of $180,000,000 and $45,000,000 respectively (B.P. Goplen - personal communication). In New Zealand, where bloat most frequently occurs in dairy cattle, up to 90 percent of the herds in a district may experience bloat, and deaths in an individual herd may exceed 15 percent.[9] The cost due to animal deaths is equivalent to Canada's. These figures do not take into account indirect losses in weight gain or milk production incurred from the cessation of feeding soon after the first symptoms of bloat appear, nor do they reflect the cost of close animal management or direct preven-

Figure 1. A victim of ruminant pasture bloat. This animal died from ingesting the alfalfa in the background. Note the distension or bloated appearance.

tative measures taken to reduce the risk of bloat, particularly in a dairy situation. The fear of bloat also reduces the utilization of nitrogen-fixing legumes as pasture forages thereby increasing input costs for nitrogen fertilizers.

WHAT IS BLOAT?

Ruminant animals have the ability to digest large amounts of fibrous plant feedstuffs. To accomplish this, they carry an active population of bacteria, fungi, and protozoa in the forestomach of their digestive system. During digestion, these microorganisms produce large amounts of gas (12-27 L/min in steers fed fresh alfalfa[1]), which will rise and separate from the mass of solid/liquid ingesta forming a free gas pocket at the top of the rumen (fig. 2). The continued expansion of this area initiates an eructation (belching) mechanism allowing expulsion of the gas through the esophagus. In normal animals, eructation occurs about once every minute, a rate sufficient to expel the large volumes of gas produced. Pasture bloat is characterized as a frothy bloat; i.e., the partially digested feedstuffs form a frothy complex that traps newly produced gas in very small bubbles throughout the entire rumen. As more gas is produced, the froth continues to expand, eventually blocking off the opening to the esophagus such that eructation cannot take place. An animal is considered to be bloating when the eructation mechanism is impaired or inhibited and the rate of gas production exceeds the animal's ability to expel the gas.[1] Such is the case when the esophagus is blocked by the frothy contents, and since large volumes of gas are produced in the rumen, the onset of bloat can occur very quickly. The outward signs of bloat in a cow include a noticeable outward distension of the flank beginning on the left and eventually progressing to both sides (fig. 3). As the condition worsens, breathing becomes labored and urination occurs more frequently. In acute bloat, the animal may fall and succumb to asphyxiation due to the pressures of the rumen on the diaphragm and lungs. Pressures within the

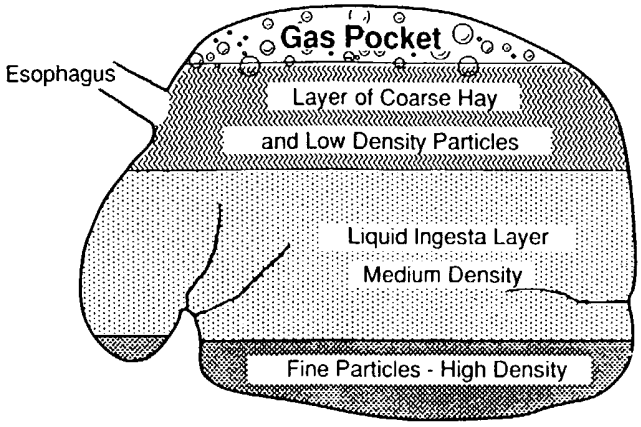

Figure 2. Diagram of the rumen or first stomach of a cow. The pocket of gas is partially emptied when rumen movements cause it to come into contact with the esophagus opening.

rumen can be very high. Removing the 6-inch diameter cap from the fistula (an artificial opening allowing access to the rumen from the side of the animal) of a bloating animal allows the soupy contents of the rumen to be ejected a distance of 8 to 10 feet (fig. 4).

ATTEMPTS TO CONTROL BLOAT

There is a high risk of bloat from pastures dominated by immature, fast-growing legumes including alfalfa and most forage clovers.[7,9] Bloat may be at least

Figure 3. Cow with a moderate to severe case of bloat. Note the distension occurring on both flanks, especially on the left.

Figure 4. Rumen contents under pressure being ejected from a cow after the opening to the fistula had been removed.

partially controlled through pasture management or the administration of anti-foaming agents.[7,9] Both attempt to minimize the chance of froth formation in the rumen. In pasture management, it would appear that controlling bloat and retaining the high nutrition offered by forage legumes could best be accomplished with non-bloating species. The bloat-safe legume forages available for pasture, however, may not be agronomically suitable for the region. Other methods of pasture management include: seeding pastures to legume:grass mixes, feeding animals to satiety on coarse roughage before pasturing, and supplemental feeding of a coarse, dry roughage. These measures attempt to reduce the proportion of bloat-causing plant material ingested to a safe level. While effective, such methods are no guarantee of bloat safety and are in some cases impractical since they require close management, a luxury that beef producers with cattle ranging over a large area do not have.

Anti-foaming agents are natural (vegetable or mineral oils, animal fats) or synthetic (pluronic-type detergents) surfactants that prevent the formation of froth in the rumen by lowering the surface tension of the foam bubbles and releasing the gas.[10] Treatments can last 12 hours or more, but to be effective, anti-foaming agents must be ingested by the animal. Methods used are: addition of these agents to feed, drinking water, or salt blocks, spraying fields to be grazed, and drenching animals causing them to lick the agent from their flanks. Such procedures are costly, labor-intensive, and necessitate adherence to a stringent management and monitoring system, making them useful only to well-controlled dairy herds.

TANNINS AND THE ETIOLOGY OF BLOAT

The currently accepted theory suggests that bloat is primarily caused by the rapid cell rupture and initial rates of digestion of ingested herbage from some

legumes[11] and grasses.[12] This theory was derived from comparisons made between bloat-causing and bloat-safe legumes. It was found that the combined effect of mechanical damage (chewing) and the rapid, early stages of rumen digestion caused greater leaf tissue disruption and mesophyll cell-wall rupture in bloat-causing legumes.[11,13] Both mechanisms contribute to bloat by allowing the rapid release of soluble leaf proteins, the principal substances responsible for the frothy nature of the rumen contents found in a bloating situation.[14] Factors identified as being responsible for the bloat-safe nature of the non-bloating legumes tested included: morphological characters such as leaf structure and venation patterns;[15] epidermal and mesophyll cell wall strength[16] and thickness;[17] and the presence of tannins in the herbage.[5,9,18]

Two non-bloating legumes, sainfoin and birdsfoot trefoil, contain tannins in their leaves. The astringent or protein-precipitating property of tannin prevents bloat by inhibiting cell wall-degrading enzymes secreted by the rumen bacteria[19,20] and rendering soluble protein in the plant cytosol immediately unavailable.[7,9] The cell rupture theory implies that retardation of the mechanical and digestive processes that promote the initial burst of cell wall rupture should inhibit the onset of bloat by making the undigested intracellular proteins unavailable for the formation of froth. Tannins by their astringency action render plants bloat-safe.

TANNINS AS A CONTROL FOR BLOAT

The bloat-safe properties of tannins were first recognized by Kendall[5] during experiments in which plant extracts from bloating and non-bloating legumes were mixed with a solvent at rumen pH and agitated in a mixer to generate a foam or froth. Extracts from the non-bloating legumes contained about 10 percent (dry wt) tannin and produced substantially less foam than the bloating legumes, which contained virtually no tannin. Foam volume in the bloating legumes was eliminated by adding commercial tannin to the extract but greatly increased when polyvinyl pyrrolidone (K-30), a compound that preferentially binds tannins[21], was added to extracts from non-bloating legumes. The inference from this study was that tannins inhibit foam production in the rumen. Kendall's work was corroborated in New Zealand where workers determined that tannins acted as protein precipitants.[22,23] As a further step, the same group conducted cattle feeding experiments using sainfoin whose herbage contained 1 to 1.5 percent of the dry matter as tannins, and red clover (*Trifolium pratense* L.) with no tannins. Bloat occurred only in those animals receiving red clover, and froth was not observed in the rumens of animals on a sainfoin diet.[22] In a more recent study, bloat occurred when cows were fed a diet of alfalfa without the addition of small percentages of dock (*Rumex obtusifolius* L.), a member of the buckwheat family. Alfalfa diets containing dock did not cause the cattle to bloat.[24] Dock has tannins in its herbage, and the amount fed to the cattle contained sufficient amounts of the secondary metabolite to precipitate both the dock and the alfalfa leaf proteins rendering the mixture of the two feeds bloat-safe.

TANNINS AND NUTRITIVE VALUE

Tannins are widely distributed in nature and are the fourth most abundant plant constituent following cellulose, hemicellulose, and lignin.[25] They don't seem to have a purely physiological function[26] but have been shown to influence growth, development, and reproduction of higher plants by interacting with auxin and gibberellic acid.[25,27] A study using high- and low-tannin strains in tissue culture showed that callus growth was greater from explants taken from the high-tannin strain.[20] It has been surmised that tannins constitute a unique quantitative defense against predators by rendering many plants repellent and unacceptable as food sources and by decreasing the dietary value of some forage crops.[26] Where some forage crops are concerned, tannins have been labelled as anti-nutritive. It has been suggested and attempts have been made to improve digestibility and palatability by reducing the tannin content in forages such as *Sericea lespedeza*[28] where tannins are responsible for the unavailability of plant crude protein,[29,30] and for low palatability in grains such as sorghum.[26] However, all tannins in herbaceous legumes are not alike. A study by Sarkar and others[31] examined six tannin-containing forage legume species and found that upon analysis of the tannins, the acid degradation products varied. A procyanidin was present in *Lespedeza cuneata* Don., an accession of Sericea, and crownvetch (*Coronilla varia* L.), both reputed to have low nutritive values, but was not detected in sainfoin or birdsfoot trefoil, forages considered to be palatable and not anti-nutritive. Further, the tannins in some species actually enhance nutrition in ruminants. Much protein from highly-digestible feed is lost by microbial degradation and subsequent absorption as ammonia in the rumen. *In vitro* and *in vivo* studies have shown that some plant tannins, if not in excess, reduce the amount of ammonia produced and make the tanned proteins available for enzymatic digestion beyond the rumen, improving the efficiency of protein utilization.[22] This is only possible if a stable tannin-protein complex is formed that does not affect the metabolism of microorganisms and does not interfere with subsequent enzymic digestion of protein in the lower gut. The tannins present in sainfoin appear to fulfill these requirements.[32,33]

TANNINS IN SAINFOIN

Sainfoin is a non-bloating perennial forage legume used for pasture and hay, but it is short-lived, intolerant of grazing, and generally less hardy than alfalfa.[1] From the pasture bloat aspect, the main interests in sainfoin lie in the bloat-inhibiting nature as well as the palatability and lack of anti-nutritive features of this legume. For a better understanding the relationship between bloat and tannins, the tannins inherent in the various species should be studied. A literature review revealed that there has not been a plethora of research carried out on the tannins found in sainfoin. This secondary metabolite was first noted in sainfoin leaves by bloat researchers in the early 1970's.[34] Sarkar and Howarth carried out a more in-depth study on the nature of the tannins in sainfoin and found that the leaves and stems contained catechin and epicatechin monomers and polymers whose acid degradation products were cyanidin and delphinidin.[31] Also, 10 species of *Onobrychis* including

sainfoin were examined for the presence of tannins with the vanillin-HCl spot test. All tested positive, and sainfoin was found to contain tannins in its leaves, flower petals, and seed coat.[35] Since then, many minor, unpublished studies have been carried out on the qualitative and quantitative presence of tannins in sainfoin organs and tissues, a number of which will be reported here.

DETECTION OF TANNINS

The vanillin-HCl method has been widely accepted for the detection of tannins in forage crops and other agricultural products[36,37] and more recently as a histochemical stain for taxonomic and physiological studies.[38] The reaction taking place in this method is the protonation of vanillin in acid solution, giving a weak electrophilic radical that reacts with the flavonoid ring of the proanthocyanidin monomer at the 6 or 8 position. This intermediate compound is dehydrated to give a red-colored chromophore.[39] The specificity of this test was questioned by Sarkar and Howarth[40] who found the vanillin reaction to be not completely specific for flavanols. Since anthocyanins will show a false positive reaction, they modified the procedure by using a reagent including methanol or ethanol with acid, but no vanillin, as a control stain to avoid a false interpretation when anthocyanins are present. This was later corroborated by Price and others.[41]

All qualitative and quantitative studies in our laboratory used the vanillin-HCl assay with the control stain or blank. For histological detection and identification of tannins, the reagent used was made up of 2 volumes of 10 percent vanillin in ethanol and 1 volume of concentrated HCl. The control solution contained the same proportions of ethanol and HCl, but minus the vanillin.[40] The vanillin stain and the control were originally applied separately to identical tissues to guard against a false positive test from anthocyanins. Later, it was found that the control stain applied to tissues could, after a suitable waiting period for any color development, be replaced by the vanillin solution. Any tannins in that same tissue produced a characteristic cherry-red color. With this method, the presence of anthocyanins could easily be differentiated both spatially and through color differences since they produced a darker red color (fig. 5). To survey large numbers of plants for the presence of tannins, the spot test[40] was used. New, fully developed leaves from the apex of the plant were crushed by a hammer between two layers of Whatman No. 3 chromatography paper. The vanillin solution was applied to the imprint of plant sap on one layer of paper and the control solution to the other to avoid the possibility of a false positive reaction. This method proved to be effective and time-saving. A number of staining methods have been tried to histologically identify tannin-filled cells, but the vanillin-HCl stain has been the most useful for the qualitative work. Other staining methods include: Safranin red and fast green; glutaraldehyde; glutaraldehyde and toluidine blue O; osmium tetroxide (OsO_4); OsO_4 and sudan black O; and OsO_4 and safranin red.

The quantitative assay for tannins was modified from Burns.[36] Either fresh or freeze-dried tissue was used although the former was preferred with some species because of noticeable discoloration of the extract and a reduction in assay values after freeze-drying. Tissue was ground in 1 percent HCl in methanol on ice using

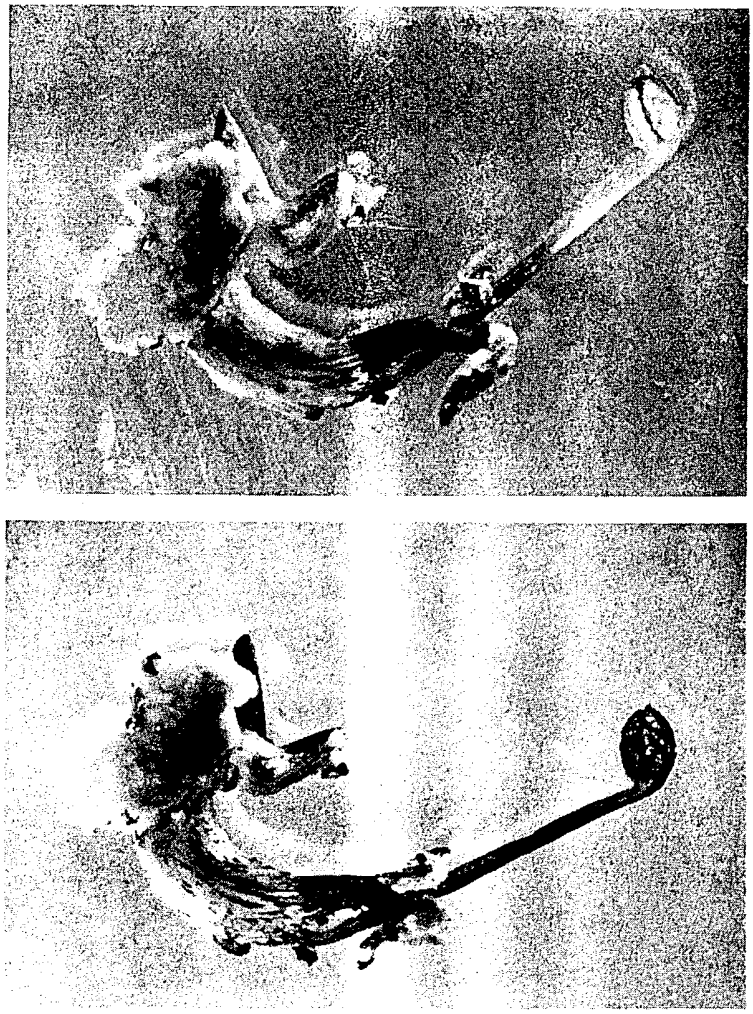

Figure 5. Sainfoin embryos derived from tissue culture and stained with the control solution (a), followed by application with the vanillin stain (b). The dark areas on the vanillin-stained embryo are tannin-filled cells (X3.3). Photographs reduced 80 percent.

a homogenizing mixer, extracted for 3 hours at 4 °C, centrifuged, and decanted. The assay[42] used control and reagent blanks with a catechin standard. Quantitatively, sainfoin leaves contain about 4 to 5 percent tannin on a dry weight basis, using the vanillin-HCl assay with catechin as a standard (our results, unpublished). Other studies using sainfoin have found the tannin content to be 1.5 percent[22] and 6 percent.[33] The amount of tannin is not constant throughout the herbage in the sainfoin plant. The pinnately compound leaf has a tannin content that increases within the individual leaflets as one proceeds along the rachis toward the leaf tip (unpublished results). No tannins have been detected in the roots, but they have been found in the flower petals and seed coat.[36] Studies in our laboratory show that most of the tannin in sainfoin is located in the leaf tissue. Viewing the sainfoin

leaf from the adaxial surface reveals a large number of microscopic clear areas that appear to extend through the leaf (fig. 6). These are the tannin sacs or vacuoles, which begin in the adaxial subepidermal layer, extend through the pallisade cells and into the spongy mesophyll tissue. When applying the vanillin-HCl stain to fresh sainfoin leaf cross-sections, the cells containing tannins appear quite striking in their vivid cherry-red coloration (fig. 7a), although they are somewhat enlarged when compared to those seen in unstained fresh cross sections or sections fixed with glutaraldehyde (fig. 7b). The same cross section reveals a subepidermal layer at the abaxial leaf surface that also stains positive with the vanillin-HCl stain. When the abaxial epidermis is removed and stained, the outline of a meshlike network of red-colored cells appears on the inner surface of the epidermis (fig. 8). Sainfoin stems in cross-section show tannins to be located in the subepidermal cells almost as a continuous layer around the periphery of the stem, dispersed among the large ground parenchyma cells located in the interfascicular regions between the vascular bundles, and in cells among the xylem tissue within the vascular bundles. The amount of tannin present in the flower was much reduced relative to that found in the leaf and stem tissure, however, tannin-filled cells were seen at the base of the sepals, ovary, and petals, along the interlocking zone of the keel petals, in the proximity of the petal veins, and within the anthers.

ONTOGENY OF TANNINS IN SAINFOIN

Using the vanillin-HCl spot test, tannins in sainfoin have been detected as early as 1 to 4 days after germination in the stems above the cotyledons and in the first and second true leaves.[42] To further examine tannin formation in sainfoin seedlings, shoot apices were fixed with glutaraldehyde and OsO_4, dehydrated with acetone, and embedded in an epon-araldite epoxy. This preparation allowed samples to

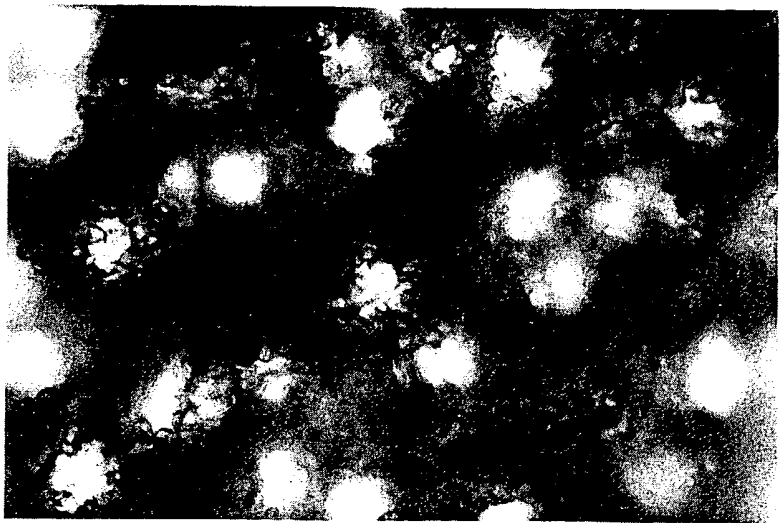

Figure 6. Sainfoin leaf viewed from the abaxial surface showing the numerous tannin sacs as clear areas (X20). Photograph reduced 80 percent.

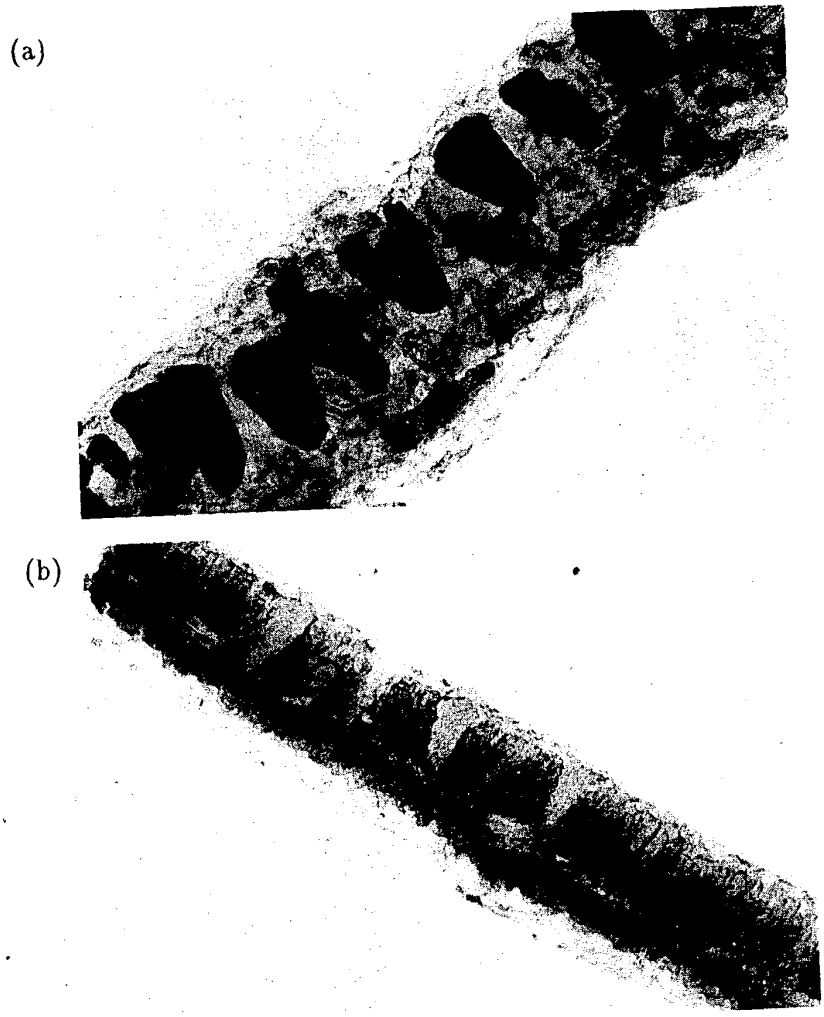

Figure 7. Fresh cross-section of a sainfoin leaf stained with vanillin-HCl (a) and fixed with glutaraldehyde (b). Note the size distortion of the tannin sacs (dark areas) in the vanillin-stained cross-section (X40). Photographs reduced 80 percent.

be thin-sectioned and stained with safranin red for initial perusal using light microscopy. Tannins are osmophilic and electron dense and are revealed as dark areas, whereas, the remainder of the cell is lightly stained with safranin red to differentiate the various organelles. Samples of potential interest are then ultra thin-sectioned, mounted on grids, stained with uranyl acetate and lead citrate, and viewed with an electron microscope.

Four day-old seedlings with fully-developed cotyledons and emerging true leaves were prepared as above. Examination of light micrographs from serial sections of the shoot apex did not reveal any cells with tannins in the apical meristem, but

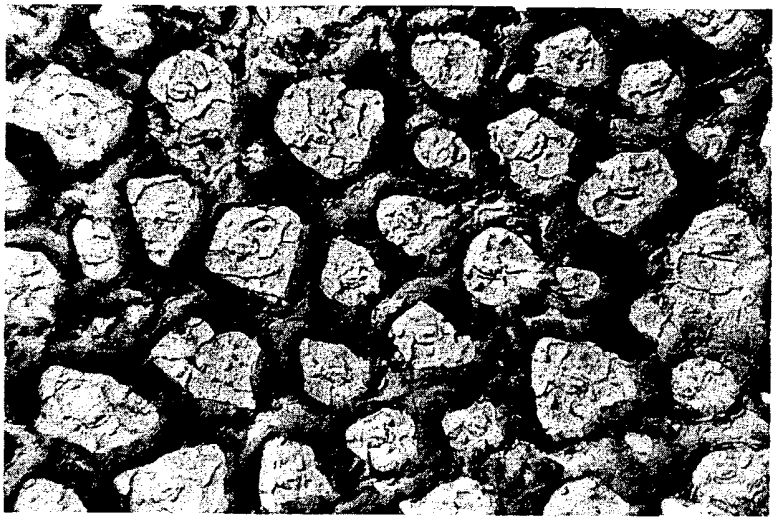

Figure 8. Abaxial epidermis removed from a sainfoin leaf and stained with the vanillin-HCl stain showing the network of tannin-staining cells (X40). Photograph reduced 80 percent.

there was a subepidermal layer in the adjacent leaf primordia with small electron dense particles in the cells that gave a hint of the earliest tannin formation. Light and electron micrographs of emerging leaves show tannins appearing on inner vacuolar surfaces of subepidermal cells in the abaxial surface of emerging leaves (fig. 9). No tannins were forming near the adaxial surface at this point, although they became evident as the leaves developed further. If tannins are a defensive mechanism, their initial presence in the abaxial cell layer might be expected since it affords protection to the exposed portion of the new folded leaflet. As the leaf

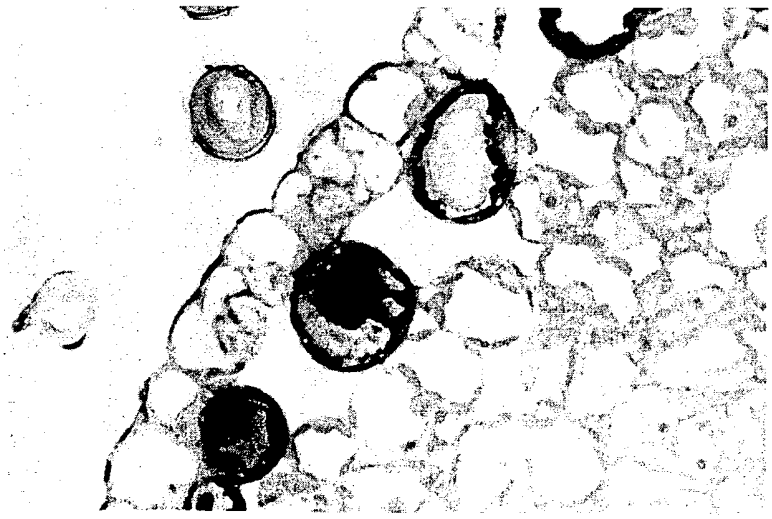

Figure 9. Cross-section of the abaxial surface of a newly emerging folded sainfoin leaf stained with osmium and safranin red (see text). The dark areas in the cell vacuoles are tannins (X400). Photograph reduced 80 percent.

develops and matures, the tannin content increases. As previously reported, electron micrographs show tannin formation occurring mainly at the periphery of the vacuole,[43,44,45,46] particularly in the early stages (fig. 10a). Numerous electron-dense particles were scattered throughout the vacuole and along the edges of the condensed tannin formations but, unlike the earlier observations,[45,46] we could not discern tannins associated with the rough endoplasmic reticulum in the cytoplasm. There were, however, membranous vesicles containing particulate matter located in the large vacuole (fig. 10b). It would appear as though the electron-dense

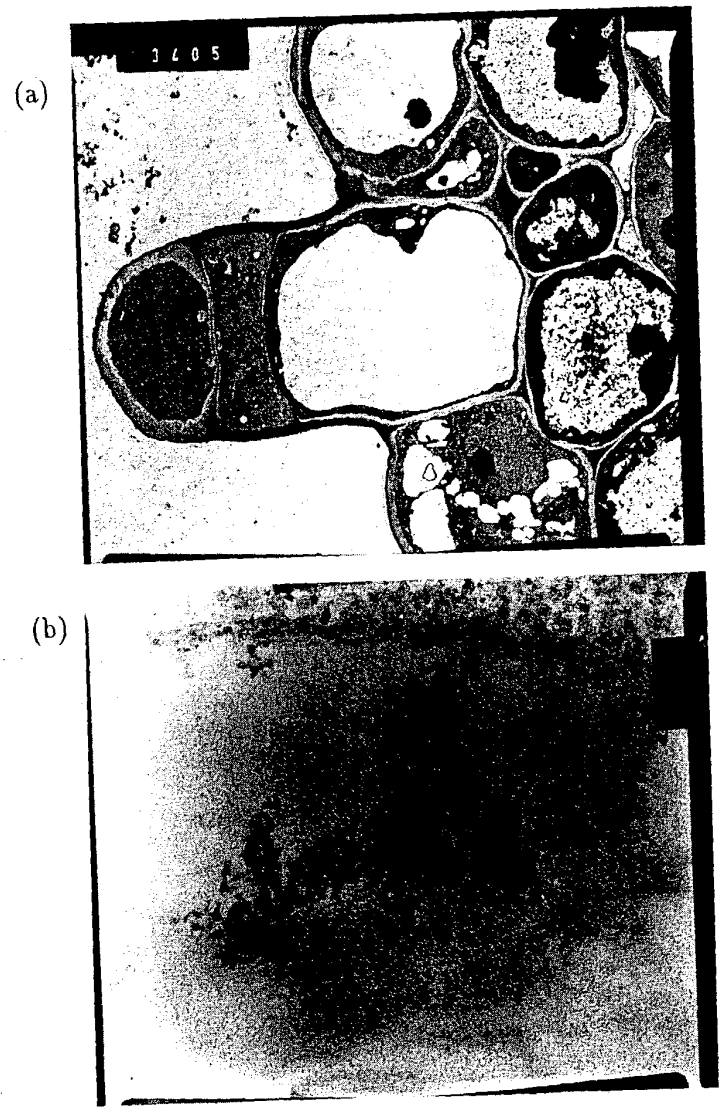

Figure 10. Electron micrograph of a newly emerging folded sainfoin leaf (a) showing the tannin-containing subepidermal cells (black areas) at the adaxial surface (X3.3K) and (b) membranous vesicles with osmium-stained particles in the vacuole (X10.5K). Photographs reduced 80 percent.

particulate matter in the vacuole is migrating to the tonoplast, or wherever tannin is forming, and enlarging the tannin mass. Mature leaves have tannin-filled cells in both subepidermal layers (fig. 11).

TANNINS IN BIRDSFOOT TREFOIL

Birdsfoot trefoil is a perennial, non-bloating legume used for pasture, hay, and silage. It is not agronomically well suited for dryland cultivation in the prairies. As in sainfoin, birdsfoot trefoil contains tannins in the leaf and stem tissue[47] but in lesser and more variable amounts. The sample we assayed had less than 1 percent of the dry weight as tannin. Others have found a range from 0.2 to 3.0 percent.[33] Leaf cross-sections show the tannins located in specific cells sporadically located throughout the mesophyll. Unlike sainfoin, there is no definite pattern of subepidermal cells or regularly arranged sacs, and when viewed from the adaxial surface, the tannin-filled cells are sometimes grouped in small clusters near the midrib. Tannins appear in trefoil seedlings from 4 to 20 days after germination,[42] about the time the first true leaves are forming. The variability of tannin content in this legume allowed the breeding of high and low tannin strains. The formation of tannins in birdsfoot trefoil appears to be controlled by a single dominant gene.[42]

TISSUE CULTURE TECHNIQUES TO INCREASE TANNIN IN ALFALFA HERBAGE

Alfalfa is known as the 'queen of the forage crops.' It enjoys worldwide adaptation and is regarded as one of the most nutritious forage legumes. Alfalfa is also known to have a high bloat potential that precludes its extensive use as a pasture crop. There are no apparent tannins in alfalfa leaves, stems, roots, or flowers. However, in all plants tested, the seeds contain tannin in the palisade cell layer of the

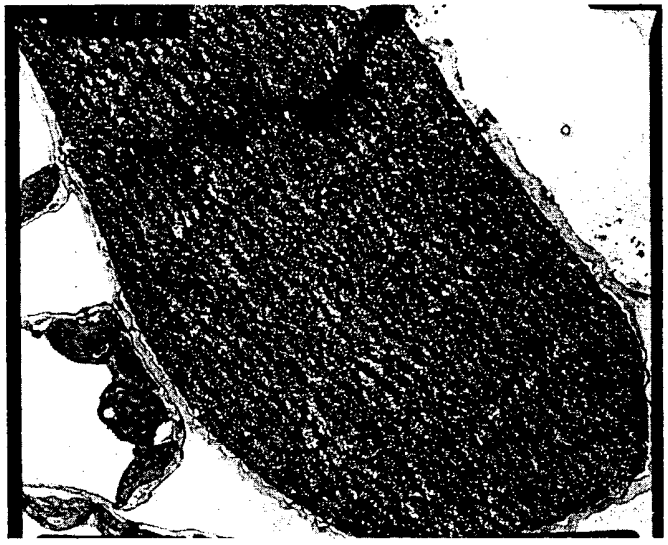

Figure 11. Electron micrograph of a mature sainfoin leaf showing a large tannin sac near the adaxial epidermis (X3.3K). Photograph reduced 80 percent.

testa.[40] The purpose of tannins in plant seed coats has been previously described.[48] The presence of tannins in one part of the plant would imply that the genetic information for tannin production is prevalent throughout the remainder. In other legume species, tannin synthesis appears to be controlled by one or two dominant genes.[37,42,49,50]

In anticipation of finding a leaf tannin-containing mutant, over 35,000 plants from 28 *Medicago* perennial and annual species were tested with the vanillin-HCl spot test. These plants were normal, mutagen-treated, tetraploid, and diploid. No tannins were found in the herbage of any of the plants tested.[40,51] The futility of this approach caused researchers to search for other methods of procuring an alfalfa plant with leaf tannins. Tissue culture offered protocols, such as somaclonal variation, which could take advantage of alfalfa's inherent potential to produce leaf tannins. Somaclonal variation is a phenomenon discovered particularly in callus and suspension culture whereby not all regenerated plants are clones of the parent as was originally perceived. It can be defined as genetic variability generated during tissue culture.[52] This variability is more frequent in culture than in nature, an observation borne out by studies in our laboratory whereby hundreds of red clover plants were regenerated through callus and suspension, revealing numerous morphological changes and chromosomal number aberrations.

With this in mind, a long-term program was designed to regenerate alfalfa plants from explant tissue through a 4-8 week callus phase. Alfalfa cotyledons from sterile seedlings were cut in two and plated in 55 mm diameter petri dishes containing B5Q, a modified B5 (Gamborg) agar medium[53] with 3.96 g/L ammonium citrate in place of the ammonium sulphate, 1.0 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) and 0.5 mg/L kinetin (6-furfurylaminopurine). The plates were incubated at 25 °C under a 16/8 day/night photoperiod with 20-30 microEinsteins $m^{-2}s^{-2}$ fluorescent lighting. After 4 weeks, any embryos that had developed were transferred to MS-k, a modified MS (Murishige and Skoog[54]) medium containing 0.1 mg/L kinetin. The remaining callus was transferred to fresh B5Q. If callus failed to produce embryos after four subcultures (4 months), it was discarded. The embryos are left on MS-k for 2 to 4 weeks, then transferred to MS-0 (hormoneless MS) for shoot and root development. When plants outgrew the petri dish, they were transferred to tall, plastic containers containing SHe, a modified SH (Shenk and Hildebrandt) medium[55] that contained 1/3 the amount of sucrose. After a month of growth, the young plants were tested for tannins using the vanillin-HCl spot test. During a 2-year period, over 300 regenerated alfalfa plants were tested before obtaining positive results. There are now 10 lines that have tested positive for elevated levels of tannin in their leaves while in culture. Tannin induction appears to be environmentally controlled, since removal of the plants from the culture situation causes a disappearance of the tannins in new growth, whereas, reintroduction to culture conditions reinstates the elevated tannin levels. More lines are being sought to build up a population that may be crossed using conventional techniques in an effort to increase the leaf tannin content.

It was mentioned earlier that there are different types of tannins. All that is known about the tannin in alfalfa is that there is a cell layer in the seed coat that stains positive for tannins with the vanillin-HCl test (fig. 12), and the residue from

the seed coat tannin is cyanidin. By contrast, the residue from both the leaf and seed coat tannin in sainfoin, a palatable, nutritious legume, is delphinidin. We do not know whether this difference has any relationship to palatability or nutrition, but it is possible that if leaf tannins in alfalfa are increased sufficiently to prevent bloat, they may be totally unsuitable in terms of these plant quality factors. Studies are now underway to elucidate the physical and chemical nature of tannin found in alfalfa seed coats and leaf tissue.

IMPLICATIONS OF TANNINS IN ALFALFA

The availability of palatable, nutritive tannins in alfalfa herbage would be of benefit worldwide. Freedom from the fear of bloat would allow maximum utilization of one of the most highly adapted, drought-resistant pasture forage crops. Pastures planted with solid stands of the long-lived perennial would have twice the carrying capacity for livestock because of its high nutritional value. The latter would be enhanced through the mechanism of tannin-associated rumen bypass. An added benefit is the legume's nitrogen-fixing capability, allowing a lower input cost to maintain the alfalfa stand and subsequent crops in the same field and increasing soil tilth and fertility when used as a green manure crop.

Obtaining a variety of alfalfa with a bloat-safe character due to the presence of tannins has been questioned. The variety would be limited in its usefulness unless the tannin trait can be easily transferred through conventional crossing methods. This could most easily be accomplished if the tannin metabolic pathway is in the plant, and its regulation is controlled by one or two dominant regulatory genes. Colvin[56] has raised the question of adaptation. Research with deer browsing high-tannin leaves and twigs has shown that rapid digestion is possible because of a high concentration of proline-rich, tannin-precipating protein in their saliva.[57] Would a constant exposure to tannin over a long period of time induce the secretion of a tannin-complexing salivary protein that would negate the effect of tannin on bloat? These and likely many other questions can only be answered when an alfalfa plant containing tannins in its herbage is produced.

In an in-depth review, Schultz[58] has pointed out that on insect herbivores, tannins have diverse effects ranging from inhibitory to stimulatory. Tannins have been shown to be effective inhibitors of some insect predators that do not normally feed on tannin-containing tissues. The proposed mechanisms, including astringency, however, are many and still speculative. There is one analogy that may be worthy of note. Earlier studies equated plant proanthocyanidins with 'woodiness' in plants, hinting at a relationship between proanthocyanidin metabolism and lignification, with a possible structural role for tannins.[26] As the growing season progresses, insect predation in oak leaves declines with a concomitant increase in proanthocyanidin formation and leaf toughness. Studies show that sainfoin leaves withstand mechanical damage to a greater degree than alfalfa[16] and are immune to the alfalfa weevil *Hypera postica* Gyllenhal, a serious pest of alfalfa.[59] With tannins possibly playing a role in this defense, their inclusion in alfalfa leaves could bring about an added benefit of resistance to an insect considered to be a major problem in alfalfa crops.

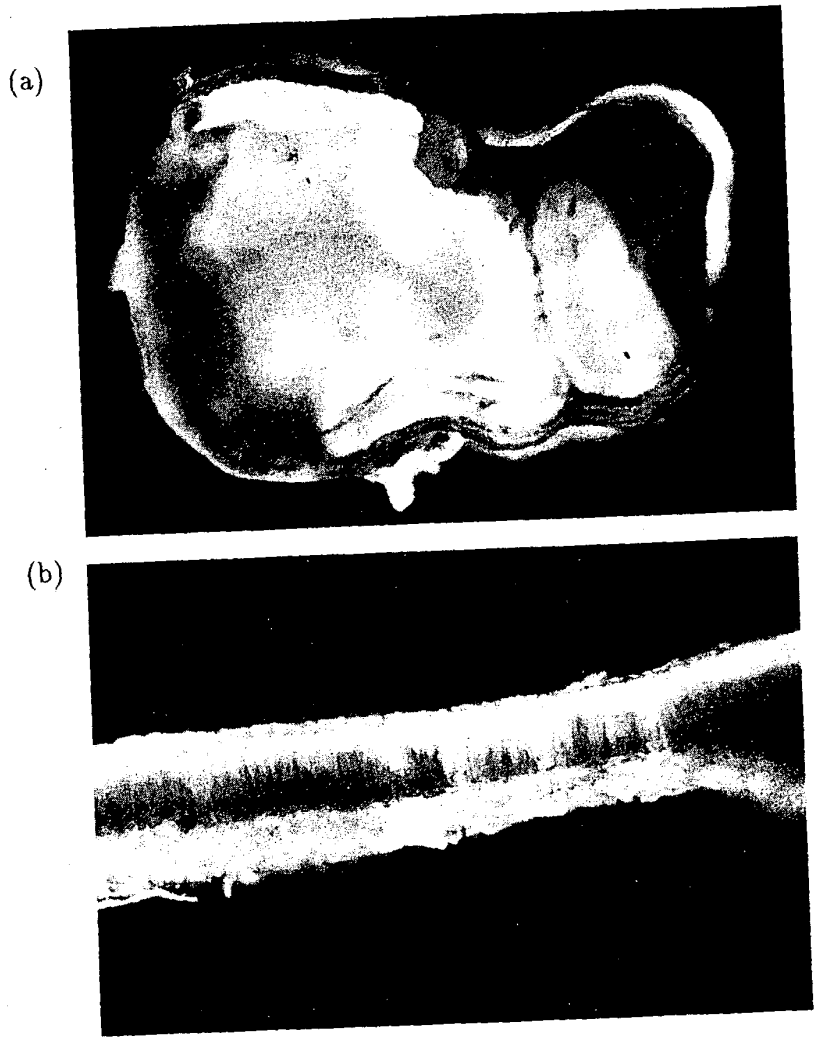

Figure 12. Transverse section of an alfalfa seed (a) stained with vanillin-HCl showing tannins in the seed coat (dark area, X16); (b) testa removed from the seed showing the dark pallisade cell layer containing tannin (X82). Photographs reduced 80 percent.

CONCLUSIONS

Sainfoin is a forage legume that has been shown to be palatable to livestock, non-bloating, and non anti-nutritive, even with its relatively high content of tannins. There is the potential to increase the tannin content in alfalfa leaves, but the tannins would likely be the same as those found in the seed coat since seed coat tissue is derived from the parent plant.[60] The ideal would be to copy the tannins from sainfoin herbage into alfalfa. Conventional breeding or tissue culture techniques do not offer the opportunity for such a transfer, but the new protocols in molecular biology and genetic engineering show promise in this direction. Before any program

on gene transfer can be initiated, much basic research is required to trace metabolic pathways and identify enzyme systems since little is known biochemically in either of these legumes. An extensive investigation will be required to find the gene(s) responsible for tannin production in sainfoin. There are many different types of tannins present in plant species, but little is known of their biological significance. An alfalfa plant with tannins in its leaves and stems would likely be bloat-safe,[3,37] but this observation is derived from inference and comparisons with other bloat-safe species. Much information is needed on the chemical structure and biochemical activity of tannins in sainfoin herbage and alfalfa seed coats. Concomitantly, the tannins present in other legumes, especially anti-nutritive species such as lespedeza, should be characterized to compare them with sainfoin tannins and perhaps identify those differences responsible for their non-nutritive nature.

REFERENCES

1. Howarth, R.E.; Chaplin, R.K.; Cheng, K.-J.; Goplen, B.P.; Hall, J.W.; Hironaka, R.; Majak, W.; Radostits, O.M. Bloat in cattle. Agriculture Canada Publication 1858/E; 34 p. (1991).
2. Davies, H.L. Review on pasture bloat. Australian Wool Corporation symposium on pasture production and utilisation. 6 p. (1985).
3. Clarke, R.T.J.; Reid, C.S.W. Foamy bloat of cattle. A review. *J. Dairy Sci.* 57:753 (1974).
4. Goplen, B.P.; Howarth, R.E.; Lees, G.L.; Majak, W.; Fay, J.P.; Cheng, K.-J. Evolution of selection techniques in breeding for bloat-safe alfalfa. *In*: Smith, J.A.; Hays, V.W. (eds.) Proc. XIV Int'l. Grassland Congress. Westview Press, Boulder, Colorado; pp. 221-223 (1983).
5. Kendall, W.A. Factors affecting foams with forage legumes. *Crop Sci.* 6:487 (1966).
6. Cole, H.H.; Boda, J.M. Continued progress toward controlling bloat. A review. *J. Dairy Sci.* 43:1585 (1960).
7. Howarth, R.E. A review of bloat in cattle. *Can. Vet. J.* 16:281 (1975).
8. Howarth, R.E. Antiquality factors and non-nutritive chemical components. *In*: Hanson, A.A.; Barnes, D.K.; Hill, R.R.Jr. (eds.) Alfalfa and alfalfa improvement. American Society of Agronomy Inc., Madison, Wisconsin; pp. 493-514 (1988).
9. Reid, C.S.W. Bloat in New Zealand cattle. *Bovine Practitioner* 1:24 (1976)
10. Kennedy, L.G. Effects of sub-clinical bloat in cattle. Dairy Science Handbook. 12:289 (1979).
11. Howarth, R.E.; Goplen, B.P.; Fesser, A.C.; Brandt, S.A. A possible role for leaf cell rupture in legume pasture bloat. *Crop Sci.* 18:129 (1978).
12. Mader, T.L.; Horn, G.W.; Phillips, W.A.; McNew, R.W. Low quality roughages for steers grazing wheat pasture. 1. Effect on weight gains and bloat. *J. Anim. Sci.* 56:1021 (1983).
13. Howarth, R.E.; Goplen, B.P.; Brandt, S.A.; Cheng, K.-J. Disruption of leaf tissues by rumen microorganisms: An approach to breeding bloat-safe forage legumes. *Crop Sci.* 22:564 (1982).
14. Howarth, R.E.; Cheng, K.-J. Fay, J.P.; Majak, W.; Lees, G.L.; Goplen, B.P.; Costerton, J.W. Initial rate of digestion and legume pasture bloat. *In*: Smith, J.A.; Hays, V.W. (eds.) Proc. XIV Int'l. Grassland Congress. Westview Press, Boulder, CO; pp. 221-223 (1983).
15. Lees, G.L.; Howarth, R.E.; Goplen, B.P. Morphological characteristics of leaves from some forage legumes: relation to digestion and mechanical strength. *Can. J. Bot.* 60:2126 (1982).

16. Lees, G.L.; Howarth, R.E.; Goplen, B.P.; Fesser, A.C. Mechanical disruption of leaf tissues and cells in some bloat-causing and bloat-safe legumes. *Crop Sci.* 21:444 (1981).

17. Lees, G.L. Cuticle and cell wall thickness: Relation to mechanical strength of whole leaves and isolated cells from some forage legumes. *Crop Sci.* 24:1077 (1984).

18. Howarth, R.E.; Goplen, B.P.; Fay, J.P.; Cheng, K.-J. Digestion of bloat-causing and bloat-safe legumes. *Annales de Recherche Veterinaires.* 10:332 (1979).

19. Goldstein, J.L.; Swain, T. The inhibition of enzymes by tannins. *Phytochem.* 4:185 (1965).

20. Lees, G.L. Condensed tannins in the tissue culture of sainfoin (*Onobrychis viciifolia* Scop.) and birdsfoot trefoil (*Lotus corniculatus* L.). *Plant Cell Reports* 5:247 (1986).

21. Anderson, J.W. Extraction of enzymes and subcellular organelles from plant tissues. *Phytochem.* 7:1973 (1968).

22. Reid, C.W.S.; Ulyatt, M.J.; Wilson, J.M. Plant tannins, bloat and nutritive value. Proc. *New Zealand Society of Animal Production* 34:82 (1974).

23. Mangan, J.L.; Vetter, R.L.; Jordan, D.J.; Wright, P.C. The effect of the condensed tannins of sainfoin (*Onobrychis viciifolia*) on the release of soluble leaf protein into the food bolus of cattle. *Proc. Nutr. Soc.* 35:95A (1976).

24. Waghorn, G.C.; Jones, W.T. Bloat in cattle 46. Potential of dock (*Rumex obtusifolius*) as an antibloat agent for cattle. *New Zealand J. Agric. Res.* 32:227 (1989).

25. Muthukumar, G.; Sivaramakrishnan, R.; Mahadevan, A. Effect of tannins on plants and their productivity. *Proc. Indian National Science Academy* B51:270 (1985).

26. Haslam, E. Plant polyphenols (syn. vegetable tannins) and chemical defense - a reappraisal. *J. Chem. Ecol.* 14:1789 (1988).

27. Mosjidis, J.A. Response to *Sericea lespedeza* genotypes low in tannins to different environments. *Plant Breeding* 101:302 (1988).

28. Donnelly, E.D.; Anthony, W.B. New Auburn Sericea variety 'Au Lotan', low tannin, nutritious forage. *Highlights of Agric. Res.* 27(2), (1980).

29. Donnelly, E.D.; Anthony, W.B. Relationship of tannin, dry matter digestibility, and crude protein in *Sericea lespedeza*. *Crop Sci.* 9:361 (1969).

30. Donnelly, E.D.; Anthony, W.B. Relationship of *Sericea lespedeza* leaf and stem tannin to forage quality. *Agron. J.* 65:993 (1973).

31. Sarkar, S.K.; Howarth, R.E.; Goplen, B.P. Condensed tannins in herbaceous legumes. *Crop Sci.* 16:543 (1976).

32. Jones, W.T.; Mangan, J.L. Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* Scop.) with fraction I leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *J. Sci. Food Agric.* 28:126 (1977).

33. Barry, T.N.; Reid, C.S.W. Nutritional effects attributable to condensed tannins, cyanogenic glycosides and oestrogenic compounds in New Zealand forages. *In*: Barnes, R.F.; Minson, D.J.; Brougham, R.W. (eds.) Forage legumes for energy-efficient animal production. USDA/CSIRO/DSIR, Palmerston North, N.Z.; pp. 1-27 (1984).

34. Jones, W.T.; Lyttleton, J.W. Bloat in cattle. XXXIV. A survey of legume forages that do and do not produce bloat. *N.Z. J. Agric. Res.* 14:101 (1971).

35. Goplen, B.P.; Howarth, R.E.; Sarkar, S.K.; Lesins, K. A search for condensed tannins in annual and perennial species of Medicago, Trigonella, and Onobrychis. *Crop Sci.* 20:801 (1980).

36. Burns, R.E. Methods of tannin analysis for forage crop evaluation. Georgia Agric. Exp. Stn. Tech. Bull. N.S. 32 (1963).

37. Marshall, D.R.; Broue, P.; Munday, J. Tannins in pasture legumes. *Aust. J. Exp. Agric. Anim. Husb.* 19:192 (1979).

38. Gardner, R.O. Vanillin-hydrochloric acid as a histochemical test for tannins. *Stain Technol.* 50:315 (1975).

39. Ribereau-Gayon, P. Plant phenolics. Oliver and Boyd, Edinburgh. (1972).

40. Sarkar, S.K.; Howarth, R.E. Specificity of the vanillin test for flavanols. *J. Agr. Food Chem.* 24:317 (1976).

41. Price, M.L.; Van Scoyoc, S.; Butler, L.G. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *J. Agric. Food Chem.* 26:1214 (1978).

42. Dalrymple, E.J. The inheritance of tannins in birdsfoot trefoil (*Lotus corniculatus* L.) and tannin development in several forage legumes. Department of Crop Science, University of Saskatchewan [Masters thesis], (1982).

43. Morrall, P.; Liebenberg, N.; Glennie, C.W. Tannin development and location in bird-resistant sorghum grain. *Scanning Electron Microscopy* 111:571 (1981).

44. Rao, K.S. Fine structural details of tannin accumulations in non-dividing cambial cells. *Ann. Bot.* 62:575 (1988).

45. Parham, R.A.; Kaustinen, H.M. On the site of tannin synthesis in plant cells. *Bot. Gaz.* 138:465 (1977).

46. Baur, P.S.; Walkinshaw, C.H. Fine structure of tannin accumulations in callus cultures of *Pinus elliotti* (slash pine). *Can. J. Bot.* 52:615 (1974).

47. Esau, K. The stem: Primary state of growth. *In*: Esau, K. (ed.) Anatomy of seed plants. John Wiley and Sons, New York; pp. 257-294 (1977).

48. Chalker-Scott, L.; Krahmer, L. Microscopic studies of tannin formation and distribution in plant tissues. *In*: Hemingway, R.W.; Karchesy, J.J. (eds.) Chemistry and significance of condensed tannins. Plenum Press, New York; pp. 345-368 (1988).

49. Picard, J. Survey of the inheritance of the character absence of tannins in seeds of broad-bean. *Annales de L'Amelioration des Plantes* 26:101-106 (1976).

50. Crofts, H.J.; Evans, L.E.; McVetty, P.B.E. Inheritance, characterization and selection of tannin-free fababeans (*Vicia faba* L.). *Can. J. Plant Sci.* 60:1135-1140 (1980).

51. Rumbaugh, M.D. The search for condensed tannins in the genus Medicago. *Am. Soc. Agron. Abstr.*, p. 75 (1979).

52. Scowcroft, W.R. Somaclonal variation: the myth of clonal uniformity. *In*: Hohn, B.; Dennis, E.S. (eds.) Genetic flux in plants. Springer-Verlag, New York; pp.216-245 (1985).

53. Attanasov, A; Brown, D.C.W. Role of genetic background in somatic embryogenesis in Medicago. *Plant Cell Tissue Organ Culture* 3:149 (1984).

54. Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473 (1962).

55. Schenk, R.U.; and Hildebrandt, A.C. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Can. J. Bot.* 50:199 (1972).

56. Colvin, H.W.; Backus, R.C. Mini Review: Bloat in sheep (*Ovis aries*). *Comp. Biochem. Physiol.* 91A:635 (1988).

57. Robbins, C.T.; Hanley, T.A.; Hagerman, A.E.; Hjelford, O.; Schwartz, C.C.; Mautz, W.W. Role of tannins in defending plants against ruminants: reduction in protein availability. *Ecology* 68:98 (1987).

58. Schultz, J.C. Tannin-insect interactions. *In*: Hemingway, R.W.; Karchesy, J.J. (eds.) Chemistry and significance of condensed tannins. Plenum Press, New York; pp. 417-433 (1988).

59. Hanna, M.R.; Cooke, D.A.; Smoliak, S.; Goplen, B.P.; Wilson, D.B. Sainfoin for Western Canada. Agriculture Canada Bulletin No. 1470 (1977).

60. Bewley, J.D.; Black, M. Seeds: Germination, structure, and composition. *In*: Bewley, J.D.; Black, M. (eds.) Seeds - physiology of development and germination. Plenum Press, New York, pp.1-27 (1985).

olento Oficial del Estado 1988 Real Decreto 475/1988 de 13 mayo, por el que se establecen los límites máximos permitidos de las aflatoxinas B$_1$, B$_2$, G$_1$, G$_2$, en alimentos para consumo humano. *BOE* 121 15325.
urley R W, Vedhara D V 1989 *The Avian Egg. Chemistry and Biology*. Wiley-Interscience Publication, New York, USA.
ampbell T C, Hayes J R 1976 The role of aflatoxin metabolism in its toxic lesion. *Toxicol Appl Pharmacol* 35 199–222.
aster W O, Burton T A, Irvin T R, Tanner M A 1986 Dietary aflatoxin, intelligence and school performance in southern Georgia. *Int J Vit Nutr Res* 56 291–295.
ies C, Pearson A M, Coleman T H, Gray J I, Pestka J J, Amin S D 1984 Tissue deposition and clearance of aflatoxins from broiler chickens fed a contaminated diet. *Food Chem Toxicol* 22 443–451.
bipley J R, Mabee M S, Applegate K L, Dreyfuss M S 1976 Further characterization of tissue distribution and metabolism of [^{14}C] aflatoxin B$_1$ in chickens. *Appl Microbiol* 28 1027–1029.
hu P S 1982 Recent progress on analytical techniques for mycotoxins in feedstuffs *J. Anim Sci* 70 3930–3963.
rpada V, Guitard R, Arbolet M 1994 Quantitative determination of aflatoxin B$_1$ in chick liver. *Food Addit Contam* 8 163–170.
ondéndez A, Verde M T, Gascón M, Ramos J J, Gómez J, Loco D F, Chávez G 1994 Variations of clinical biochemical parameters of laying hens and broiler chickens fed aflatoxin-containing feed. *Avian Pathol* 23 37–47.
rner C, Ryder R, Mortimore R 1985 Monitoring of aflatoxin in human body fluids and application to field studies. *Cancer Res* 45 922–928.
ínez P J, Fernández C A 1988 Estudio de aflatoxicosis en gallinas ponedoras. I. Niveles de aflatoxinas en mollejas y su posible peligro para el consumo humano. *XXVI Symposium de Avicultura Científica*. Reus, Spain, pp. 413–434.
agary III J F, Mackey D B 1981 High performance liquid chromatographic determination of aflatoxins in animal tissues and products. *JAOAC* 64 144–151.
eland E C, Cardellhac P T 1975 Excretion of carbon-14-labeled aflatoxin B$_1$ via bile, urine, and intestinal contents of the chicken. *Am J Vet Res* 36 909–912.
adrickas E G 1991 Kwashiorkor: The hypothesis that aflatoxicosis aflatoxicosis. *Pediatrics* 88 376–379.
gan G R, Ryan N J, Hayes A W 1979 Aflatoxin B$_1$ and Reye's syndrome. *Lancet* I 561.
obson W C, Wiseman H G 1974 The transmission of aflatoxin B$_1$ into eggs. *Poultry Sci* 53 1742–1745.
o C A, Rump R, Kowalsky J 1989 Low level exposure of ovaries and laying hens to aflatoxin B$_1$ from naturally contaminated corn. *Arch Geflügelk* 53 204–206.
ur H, Jood S K, Joshi D V, Singh K B 1991 A short note on aflatoxins in poultry livers. *Indian Vet* 68, 955–956.
dstedt R, Leistner L 1977 Transmission of aflatoxins into eggs and egg products. *Ann Nutr Alim* 31 499–506.
ghee M S, Chipley J R 1973a Tissue distribution and metabolism of aflatoxin B$_1$-^{14}C in broiler chickens. *Appl Microbiol* 25 763–769.
ghee M S, Chipley J R 1973b Tissue distribution and metabolism of aflatoxin B$_1$-^{14}C in layer chickens. *J. Food Sci* 38 566–570.
Micco V, Miraglia R, Orsoni R, Brera C, Mantovani A L, Lopalco A, Staiotta *D* 1985 Long-term administration of low doses of mycotoxins to poultry. I. Residues of aflatoxin B$_1$ and its metabolites in broilers and laying hens. *Food Addit Contam* 5 293–308.
Nabney J, Nesbitt B F 1965 A spectrophotometric method for determining the aflatoxins. *Analyst* 90 153–160.
Neal G E 1987 Influences of metabolism: aflatoxin metabolism and its possible relationships with disease. In: *Mycotoxins in Food. Progress and Prospects*, ed Watson D H. VCH-Ellis Horwood, Chichester, UK, pp. 125–168.
Oriuchi W I, Stahr H M, Kraft A A 1986 Distribution and effects of aflatoxin in children tissue after feeding radiolabeled [^{14}C] aflatoxin B$_1$. *J Food Protect* 49 799–803.
Patterson D S P 1973 Metabolism as a factor in determining the toxic action of the aflatoxins in different animal species. *Food Cosmet Toxicol* 11 289–294.
Pier A C 1981 Mycotoxins and animal health. In: *Advances in Veterinary Sciences and Comparative Medicine* (Vol. 25). Academic Press, Inc, New York, USA, pp. 185–243.
Rajagopalan M S, Buch M P, Blum H E, Vyas G N 1985 Interaction of aflatoxin and hepatitis B virus in the pathogenesis of hepatocellular carcinoma. *Life Sci* 39 1287–1290.
Richard J L, Pier A C, Stubblefield R D, Shotwell O L, Lynn R L, Cullip R C 1983 Effect of feeding corn naturally contaminated with aflatoxin on feed efficiency, on physiologic, immunologic, and pathologic changes, and on tissue residues in steers. *Am J Vet Res* 44 1294–1299.
Richard J L, Stubblefield R D, Lynn R L, Peden W M, Thurston J R, Rimler R B 1986 Distribution and clearance of aflatoxins B$_1$ and M$_1$ in turkeys fed diets containing 10 or 150 ppb aflatoxin from naturally contaminated corn. *Avian Dis* 30 788–793.
Sawhney D S, Vadehra D V, Baker R C 1972 The metabolism of ^{14}C aflatoxins in laying hens. *Poultry Sci* 51 1302–1309.
Shotwell O L, Hesseltine C W, Stubblefield R D, Sorenson W G 1966 Production of aflatoxin on rice. *Appl Microbiol* 14, 425–428.
Stubblefield, R D 1987 Optimum conditions for formation of aflatoxin M$_1$-trifluoroacetic acid derivative. *JAOAC* 70 1047–1049.
Tebib H M, Fakhry F M 1988 Aflatoxin B$_1$ residues in broiler and its effect on fat metabolism. *Vet Med J* 36 135–145.
Trucksess M W, Stoloff L 1981 High performance liquid chromatographic determination of aflatoxicol in milk, blood, and liver. *JAOAC* 64 1083–1087.
Trucksess M W, Stoloff L 1984 Determination of aflatoxicol and aflatoxins B$_2$ and M$_2$ in eggs. *JAOAC* 67 317–320.
Trucksess M W, Stoloff L, Young K, Wyatt D, Miller B L 1983 Aflatoxicol and aflatoxins B$_2$ and M$_2$ in eggs and tissues of laying hens consuming aflatoxin-contaminated feed. *Poultry Sci* 62 2176–2182.
Wogan G N, Paglialunga S, Newberne P M 1974 Carcinogenic effects of low dietary levels of aflatoxin B$_1$ in rats. *Food Cosmet Toxicol* 12 681–685.
Wolzak A., Pearson A M, Coleman T H, Pestka J J, Gray J I, Chen C 1985 Aflatoxin deposition and clearance in the eggs of laying hens. *Food Chem Toxicol* 23 1057–1061.
Wolzak A, Pearson A M, Coleman T H, Pestka J J, Gray J I, Chen C 1986 Aflatoxin carryover and clearance from tissues of laying hens. *Food Chem Toxicol* 24 37–44.

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Effect of High Temperature on Condensed Tannin Accumulation in Leaf Tissues of Big Trefoil (*Lotus uliginosus* Schkuhr)

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Abstract: Plants from three clones of big trefoil (*Lotus uliginosus* Schkuhr) having low, medium and high concentrations of tannin in their leaves were subjected to growing conditions with normal (20°C) and high (31°C) temperature regimes. Plants were tested every 3 weeks for foliar condensed tannin content using the vanillin–HCl and the butanol–HCl assays. Plants from clones growing under high temperatures matured more quickly and by 14 days had condensed tannin levels substantially greater than plants from the same clones grown under normal temperatures. Clones remained consistent with respect to their parental tannin levels throughout two trials. Leaves from high-temperature clones suffering additional nutrient stress symptoms had very low levels of foliar condensed tannins and cross-sections of chlorotic leaves revealed no absence of large tannin vacuoles. It is concluded that high-temperature stress can induce the formation of additional condensed tannin in the leaves of this species.

Key words: big trefoil, *Lotus uliginosus*, condensed tannin, proanthocyanidins, catechin, flavanol, temperature stress.

INTRODUCTION

Big trefoil (*Lotus uliginosus* Schkuhr), also described as *L. pedunculatus* Cav (Heath 1970) is a long-lived perennial forage legume used for pasture, hay, and seed production in the pacific northwest and parts of the eastern USA, Australian and New Zealand (Kaiser and Heath 1988; Hill and Witchwood 1990) Big trefoil is known to contain condensed tannins (CTs) or flavanols in its leaves and stems (Jones *et al* 1976; Barry and Forss 1983; Lowther *et al* 1987). CTs may be an important part of the chemical defence of many plants against vertebrate and invertebrate herbivores (Haslam 1988). In recognition of this role, a number of cotton germplasm lines containing elevated levels of CTs recently were registered to provide resistance against a variety of insect and disease pests (Smith *et al* 1990). When present in the foliage of forage legumes, it is generally acknowledged that CTs are considered the agent responsible for the prevention of bloat in cattle (Guirk *et al* 1974; Ross and Jones 1974; Chiquette *et al* 1989). CTs can confer a nutritional advantage to forages such as sainfoin (*Onobrychis viciifolia* Scop) by facilitating the bypass of protein that might otherwise be lost through deamination in the rumen (Barry and Forss 1983; Barry and Duncan 1984). However, these secondary metabolites also can be detrimental to the nutritional value of forages such as *Sericea lespedeza* and big trefoil by depressing voluntary intake and digestion (Donnelly and Anthony 1985; Barry and Duncan 1984).

The amount of CTs found in the foliage may vary with genotype, but can be influenced also by seasonal changes. Higher concentrations are reported in birch and maple in late summer than in winter (Baldwin *et al* 1987). Low soil fertility and acid soil conditions can result in increased foliar levels of CTs in *Lotus* species (Ross and Jones 1974; Barry and Duncan 1984; Kolmans and Tanner 1990). Some of the positive and negative effects of CTs are related to the concentrations found in the plant material (Barry 1984), thus, the

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ability to regulate CT accumulation through selection and environmental change would allow determination of thresholds for some of the effects of CTs. Such findings could aid in breeding plant material with CT levels that would be advantageous.

Except for the seasonal changes mentioned, there have been no definitive reports on the levels of CTs in plants grown at higher temperatures. In this study, we report on the effect of two temperature regimes on CT levels in the leaves from clones of big trefoil grown under defined conditions in growth cabinets.

EXPERIMENTAL

Plant selection and growth

Plants used in this study were clones obtained from field selections of big trefoil (*Lotus uliginosus* Schkuhr). Initially, selections were made from approximately 40 field-grown plants based on low, medium and high tannin content using the vanillin–HCl spot test of Sarkar and Howarth (1976) and scored using the method of Dalrymple *et al* (1984). Two plants were selected from each of the three tannin levels and transplanted from the field into the greenhouse. Once the plants were established, enough cuttings were taken and rooted to provide 10 clones from each of the six parents. For experimental purposes during subsequent growth and development, five established clones from each parent were placed in a growth cabinet providing a 16 : 8 h photoperiod [300 μmol $m^{-2} s^{-1}$] using a combination of incandescent/fluorescent lighting, and set to a corresponding 20 : 15°C temperature regimes. The other five clones from each parent were placed in a separate growth cabinet with identical conditions except for the temperature regime of 30 : 25°C.

Experimental procedure and chemical assays

Two experiments were performed. In the first experiment, young plants were allowed to grow in their respective temperature environments for 81 days. Samples were taken and assayed using the vanillin–HCl microassay four times during this period at approximately 3-week intervals. During tests for flavan-3-ol monomers (catechin) and polymers (CTs), each clone was treated as a sample and was sub-sampled three times. To obtain a representative sample, individual leaves from the upper half of stems from all five clonal plants were picked randomly and 200 mg weighed into each of three 50 ml centrifuge tubes to which 20 ml of 4% (v/v) HCl–methanol was added immediately. At each sampling interval, a total of 1·5 g of leaves was harvested from all of the clones and dried for 72 h at 70°C for dry weight determination. Fresh samples were ground for 30 s at setting 5 using a Brinkmann Polytron® PT 10-35 homogeniser equipped with a PT 20 generator probe. Homogenates were extracted in 1% HCl–methanol at 4°C for 2 h, then centrifuged for 10 min at 600 × *g* retaining the supernatant for assay. Flavan-3-ol concentrations were determined using the vanillin–HCl assay method of Dalrymple (1982) using catechin as a standard. The data presented was obtained after adjustment to reflect values based on a standard curve using purified big trefoil CT (Muir A D unpublished).

In the second experiment, new cuttings from each clone were obtained, and after establishment for 3 weeks in the greenhouse, the plants were allowed to grow under the two temperature regimes for 170 days. Plants were harvested and the CT content determined using the butanol–HCl assay at approximately 3-week intervals for the first four assays followed by two assays 6 weeks apart. Two assays using the vanillin procedure were also performed during this trial as a check. Foliar CTs were determined using a modified version (Muir A D unpublished) of the butanol–HCl assay of Watterson and Butler (1983) which is specific to the polymer. In this assay, six to 10 stems were taken from one plant of each clone and placed in plastic bags. Two hundred mg of individual leaves chosen from the upper half of the stems were weighed out, mixed and 50 mg sub-samples in each of four 19 × 50 mm vials. An overall 1·5 g sample of leaves for dry weight determination were obtained from plants in each of the 20°C and 30°C growth cabinets. Samples were ground in 6 ml of methanol using a motor-driven glass mortar and pestle. One ml of the ground sample was pipetted into a 16 × 125 mm screw-cap test tube containing 100 mg of acid-washed polyvinylpolypyrrolidone (PVPP) (Polyclar® AT) and vortex-mixed. For a standard, 1 ml aliquots of isolated and purified big trefoil CT polymer (200 μg ml^{-1}) were pipetted into four tubes containing PVPP and mixed. Chlorophyll was removed by rinsing samples in 8 ml of methanol, mixing and centrifuging at 1000 × *g*. This process was repeated three times. The standards were washed once with methanol. Five ml of 30% (v/v) HCl–butanol was dispensed into all tubes, mixed, capped and placed in a 70°C waterbath for 1 h. After heating, the tubes were mixed again, centrifuged at 1000 × *g* for 4 min, and the supernatant filtered through no. 4 Whatman filter paper into 16 × 100 mm test tubes. The filtrate was read at 550 nm using butanol–HCl as a blank.

Microscopy

Light and electron micrographs of big trefoil leaf cross-sections were obtained following the osmium tetroxide–safranin O method of Lees *et al* (1993).

RESULTS AND DISCUSSION

Big trefoil is not a crop normally grown in the Canadian prairies, however, this species was established at Saskatoon in the spring of 1991 allowing access to over 50 individual field-grown plants. When used on individual plants, the vanillin–HCl spot test gave a quick, qualitative and semi-quantitative indication of the amount of flavolans present through differences in colour intensity and provided a rapid selection method. The variability proved to be high within the species (results not shown) allowing selection of six plants, two with low-, two with medium- and two with a high-tannin content in the leaves. These plants were the source of clonal material used in subsequent temperature stress experiments. Subsequent quantitative assays showed that the low pair had tannin values of 28 and 30 mg tannin g^{-1} DW, the medium values were 50 and 70, while the high values were 100. This variability in big trefoil CT content has been reported previously in field-grown plants by Barry and Manley (1984) and Kelman and Tanner (1990) who found tannin ranging from 46 to 106 g kg^{-1} and 25 to 107 g kg^{-1} respectively on a dry weight basis. These tannin values are consistent with our greenhouse-grown plants, however, the choice of the leaves in our subsequent sampling for assay may have resulted in a bias towards higher values because we chose green, fully expanded leaves from the upper half of the stem which contain the highest tannin content (Table 1). We have also shown this to be true for leaflets of sainfoin (unreported results).

In the first experiment, clones were grown at both temperatures for 81 days before being cut back. During the growing period the 20°C plants remained mostly vegetative with a few beginning to flower towards the end. The 30°C plants began to flower within 3 weeks of being subjected to the high temperature and plants had a greater number of stems with smaller, more numerous leaves. Table 2 shows the results of CT determinations on leaf material sampled at 3-week intervals. The data are the means of 30 plants in each temperature regime.

TABLE 1
Tannin content (mg g^{-1} DW) of leaves from the upper, middle and lower third of the stem from 60 day big trefoil plants grown from cuttings

Stem position	Tannin content* 20°C
Tip and 1st node	23·6a
2nd and 3rd node	13·4ab
4th and 5th node	11·6b

* Determined using the vanillin–HCl assay. Data adjusted using extracted CT polymer as a standard. $n = 6$, values with different letters are significantly different, $P = 0{\cdot}05$, Student's *t*-test.

TABLE 2
Tannin content (mg g^{-1} DW*) of leaf samples from big trefoil clones grown at 20 and 30°C as a function of time of growth in each temperature regime. Plants were cut back after 81 days and allowed to regrow for 42 days before the final analysis

Temperature	Time in growth cabinet (days)					
	14	33	54	81	Cut back	123
20°C	24·1a	107·7a	108·3a	119·4a	103·0a	
30°C	37·9b	152·7b	149·7b	130·3a	34·9b	

* Determined using the vanillin–HCl assay. Data adjusted using extracted CT polymer as a standard. $n = 27$–30, values with different following letters are significantly different, $P = 0{\cdot}01$, Student's *t*-test.

Differences between the two groups were evident at the first sampling date. Although the amount of CT increased with time in both temperature regimes, CT content increased more rapidly and stayed higher in the 30°C plants than in the 20°C plants up to 54 days. The differences noted between the two temperature regimes were highly significant for the three sampling dates. The 30°C values subsequently decreased as shown by the last assay at 81 days and the difference between them was not significant. At 81 days the 20°C plants appeared healthy with dark-green leaves and some plants coming into flower, however, the 30°C plants were showing major signs of heat stress including senescent lower leaves, smaller leaf area, numerous leaves with red pigmentation, elongated stems, and an abundance of flowers. The earlier senescence in the 30°C plants was probably responsible for their reduced CT content since a similar reduction has also been noted in senescing sainfoin leaves (unpublished). After the 81-day growing period both groups of plants were cut back and allowed to regrow in their respective temperature regimes for another 42 days until it was noted that the 30°C plants were not recovering as expected. The 20°C plants had good regrowth and when sampled for CT revealed concentrations lower than those obtained before the plants were cut back. The 30°C plants also regrew, but the plants were stunted and in most cases, chlorotic, some being totally yellowed and others bearing conspicuously red leaves. Since no fertiliser had been applied to these plants during the first growing period, it was likely that nutrient deficiency was additional to heat stress in causing some of the observed symptoms. The amount of tannin in the 30°C plants decreased drastically indicating that when new leaves were developing, either the tannin formation was not taking place, or that existing CT was catabolized. Histological sections revealed that whereas green leaves from plants in this group contained tannin vacuoles in both sub-epidermal layers (Fig. 1a), no typical CT vacuoles

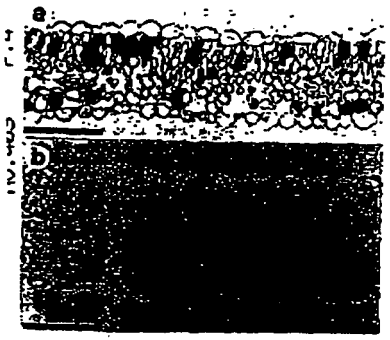

Fig 1. Light micrographs of cross-sections of big trefoil leaflets from plants which were cut back and regrown for 12 days at 30°C. CTs are shown as black areas within the subepidermal layers; (a) green leaflet showing the presence of tannin-filled cells, (b) yellow senescing leaflets showing no tannin-filled cells. Bar—100 μm.

were observed in subepidermal layer of leaves from chlorotic plants (Fig. 1b). Closer examination using transmission electron microscopy revealed cells containing small amounts of electron-dense material, probably CT, near the abaxial epidermis in these leaves (Fig. 2a and b). Why this should occur is not known, but it has been suggested that plants can exhibit plasticity in their allocation of resources. Stresses imposed by nutrient deficiencies may cause more primary than secondary metabolism during growth and CT synthesis might depend on the amount of photosynthate available (Feeny 1970). Since the majority of the plants used for CT content were chlorotic, this observation would account for the low amount of CT detected by the vanillin–HCl assay in all of the clones.

To confirm the results of the first experiment, and to determine how much of the flavanols detected by the vanillin–HCl assay were in the polymeric (CT) form, a second experiment was conducted utilizing the butanol–HCl assay as a quantitative measure for the CTs present. In this experiment the plants were fertilized once during the growing period to try and prevent the nutrient stress previously observed at 30°C. The 20°C plants appeared healthy during the entire growth period with many still flowering when the experiment terminated. The 30°C plants grew much the same as in the first experiment, but without the severe stress symptoms noted earlier. Towards the end of the 170 day growth period the 30°C plants were senescing. At 15 and 55 days, the plants were tested with both the vanillin–HCl and the butanol–HCl assays (Table 3) confirming the results from the first experiment (Table 2). CT determinations with both assays in the two temperature regimes gave the expected increase and differences between the clones. For the butanol–HCl test, differences were not only highly significant when all of the plants in one growth chamber or temperature regime were tested as a group as shown in Table 3, but also when plants with different tannin levels were tested against each other, ie the medium tannin plants from the 20°C chamber had lower tannin levels than their counterparts in the 30°C chamber, etc. The difference in vanillin–HCl CT values between the 20 and 30°C plants at 15 and 55 days was large with the latter being greater than two-fold, however, the increase in values for both temperature groups between 15 and 55 days was not as widespread as those obtained for the same time periods in the first experiment. Since these plants had been previously grown under identical conditions, it is possible that a preconditioning has taken place accounting for the higher values at 15 days. The data obtained for the

TABLE 3

Tannin content (mg g^{-1} DW) of leaf samples from all big trefoil clones grown at 20 and 30°C as a function of time of growth in each temperature regime. Assays were done using the butanol–HCl method with vanillin–HCl assays interspersed as checks

Temperature (°C)	Assay	Regrowth days[a]					
		15	57	55	93	134	170
20	Butanol–HCl	149·3a	160·9a	112·6a	135·5a	205·8a	245·6a
	Vanillin–HCl[b]	58·8a		68·2a			
30	Butanol–HCl	204·8b	231·4b	245·3b	227·4b	249·6b	205·5b
	Vanillin–HCl	82·7b		162·9b			

[a] New cuttings were allowed to grow for 3 weeks in the greenhouse, then placed in experimental cabinets. $n = 24$, values with different following letters are significantly different, $P = 0{\cdot}001$, Student's t-test.

[b] Data adjusted using extracted CT polymer as a standard.

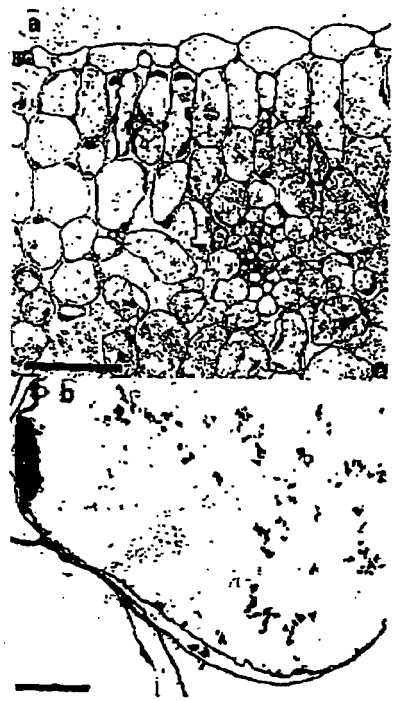

Fig 2. Electron micrographs of cross-sections of big trefoil leaflets from plants which were cut back and regrown for 12 days at 30°C; (a) finer cellular structure showing abaxial subepidermal cells with some CT in the vacuole (arrowhead), but no large adaxial sub-epidermal CT-filled cells. Bar–50 μm. (b) Magnified view of adaxial sub-epidermal cell showing CT in the vacuole. Bar—5 μm.

butanol–HCl assay and the adjusted values for the vanillin–HCl procedure for 15 and 55 days are higher, but consistent with those reported for *Lotus pedunculatus* by Terrill *et al* (1992). Values in the butanol–HCl data for the remainder of the 170-day period in both temperature regimes show consistent differences between the 20 and 30°C experiments similar to the vanillin–HCl data in the first experiment. At 170 days the values for the 30°C clones dropped, but again this is probably a reflection of a more advanced stage of senescence in these plants.

Table 4 shows the amount of tannin from clones in each of the low, medium and high categories in trial one. Values for the 31- and 54-day periods show highly significant differences when comparing two temperatures within the three tannin levels chosen. It is interesting to note that the differences in the amount of CT between the three groups remained consistent throughout the experiment indicating that the expression of CT among these cloned lines is genetically controlled.

The quantitative data in the present study demonstrates that in leaves of big trefoil high concentrations of CT on a dry weight basis is consistent with a report by Levin (1976) which states that CT in many species of plants often exceeds 10%. The validity of the temperature stress data was confirmed by viewing histological sections. The high amount of CT present is demonstrated in Fig 3 which shows cross sections of leaves from high-tannin clones grown at 20°C and 30°C after 81 days. Although the tannin vacuoles are not completely filled with CT, their frequency and size is consistent with a maximum tannin concentration at 54 days. Partially filled vacuoles also are consistent with the quantitative data at 81 days showing a reduction in CT for the clones grown at 30°C.

The present study demonstrates that there is a stress effect due to elevated temperatures resulting in higher concentrations of flavolan monomers and polymers in the leaves of big trefoil. Although the increase may have been influenced by nutrient stresses in the first experiment, similar effects were observed in the second experi-

Fig 3. Light micrographs of cross-sections of big trefoil leaflets after 81 days of growth in temperature-controlled growth cabinets. CTs are shown as black areas within the subepidermal layers; (a) grown at 20°C, (b) grown at 30°C. Bar—100 μm.

TABLE 4

Tannin content (mg g^{-1} DW*) of leaf samples from clones of low, medium and high tannin field selections grown at 20 and 30°C. Plants were cut back after 21 days and allowed to regrow for 42 days before being the final analysis

Tannin content	Temperature (°C)	Time in growth cabinet (days)				
		14	35	54	81 Cut back	123
Low	20	18·2a	85·0a	86·0a	102·6a	92·2a
	30	22·9a	128·7b	129·3b	104·9a	33·4b
Medium	20	22·1a	100·1a	118·7a	115·9ab	107·2c
	30	43·1b	155·1c	147·7bd	137·2ab	21·3bd
High	20	32·4b	138·7b	129·3c	139·4c	11·6c
	30	43·6b	173·4c	172·9f	155·5d	56·6d

* Determined using the vanillin–HCl assay. Data adjusted using extracted CT polymer as a standard. $n = 8$–10; values with different following letters are significantly different, $P = 0.05$, Student's t-test.

ment when nutrient deficiencies were alleviated. Stress has been shown to be a factor in increasing a number of secondary metabolites, including CTs in plants. In tissue culture, stress in the form of single nutrient deficiencies, mainly phosphorous, results in elevated concentrations of CT (Mantell and Smith 1983; Gershenson 1984). In big trefoil, Barry and Forss (1983) reported a reduction in the concentration of CT when nutrient deficiencies are corrected, and a similar response is noted in some cultivars when the pH is raised through the addition of lime (Kelman and Tanner 1990). There is an implication of CT induction as a result of temperature increase in leaves of *Acer* (Baldwin *et al* 1987) and *Quercus* (Feeny and Bostock 1968); this is a seasonal response occurring between spring and summer. Leaves of *Sericea lespedeza* also accumulate CT between May and July (Cope *et al* 1971). It is clear from the present study that there is a strong temperature effect in CT accumulation in *L uliginosus*. This knowledge could be utilised in experiments requiring manipulation of CT concentrations; for example, to determine effective thresholds of CT within big trefoil or possibly other species as a defence against herbivores or pathogens.

Further studies will be directed at elucidating such interactions, and in testing the proposition that temperature-induced CT accumulation is a general, rather than a specific phenomenon in tannin-expressing herbaceous legumes.

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REFERENCES

Baldwin I T, Schultz J C, Ward D 1987 Patterns and sources of leaf tannin variation in yellow birch (*Betula allegheniensis*) and sugar maple (*Acer saccharum*). *J. Chem Ecol* 13 1065–1078.

Barry T N 1984 The role of condensed tannins in the digestion of fresh *Lotus pedunculatus* by sheep. *Can J Anim Sci* 64 (Suppl) 181–182.

Barry T N, Duncan S J 1984 The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 1. Voluntary intake. *Br J Nutr* 51 485–491.

Barry T N, Forss D A 1983 The condensed tannin content of vegetative *Lotus pedunculatus*, its regulation by fertilizer application, and effect upon protein solubility. *J Sci Food Agric* 34 1047–1056.

Barry T N, Manley T R 1984 The role of condensed tannins in the nutritional value of *Lotus pedunculatus* for sheep. 2. Quantitative digestion of carbohydrates and proteins. *Br J Nutr* 51 493–504.

Chiquette J, Cheng K-J, Rode L M, Milligan L P 1989 Effect of tannin content in two isosynthetic strains of birdsfoot trefoil (*Lotus corniculatus* L) on feed digestibility and rumen fluid composition in sheep. *Can J Anim Sci* 69 1031–1039.

Cope W A, Bell T A, Smart W W G 1971 Seasonal changes in an enzyme inhibitor and tannin content in *Sericea lespedeza*. *Crop Sci* 11 893–895.

Dalrymple B J 1982 The inheritance of tannins in birdsfoot trefoil (*Lotus corniculatus* L) and tannin development in several forage legumes. Master's thesis, University of Saskatchewan, Canada, p 14.

Dalrymple E J, Goplen B P, Howarth R E 1984 Inheritance of tannins in birdsfoot trefoil. *Crop Sci* 24 921–923.

Donnelly E D, Anthony W B 1983 Breeding low-tannin cultivars. IX. Variation in forage quality factors among lines. *Crop Sci* 23 982–984.

Feeny P P, Bostock H 1968 Seasonal changes in the tannin content of oak leaves. *Phytochemistry* 7 871–880.

Gershenson J 1984 Changes in the levels of plant secondary metabolites under water and nutrient stress. In: *Recent Advances in Phytochemistry* (Vol. 18 Phytochemical Adaptations to Stress), eds Timmermans G N, Sondheimer C & Loewus F A. Plenum Press, New York, USA.

Goplen B P, Goplen B E, Howarth R E, McArthur J M 1974 Variation of soluble proteins in alfalfa, sainfoin, and birdsfoot trefoil. *Crop Sci* 14 495–499.

Haslam E 1988 Plant polyphenols (syn vegetable tannins) and chemical defence—a reappraisal. *J Chem Ecol* 14 1789–1805.

Heath M E 1970 Naturalized big trefoil (*L pedunculatus* Cav) ecotype discovered in Crawford County, Indiana. *Proc Indiana Acad Sci* 79 193–197.

Hill M J, Wilkinson S 1990 Vegetative and reproductive development in *Lotus uliginosus* Schk. cv 'Grasslands Maku'. *Appl Agric Res* 5 169–175.

Jones W T, Broadhurst R B, Lyttleton J W 1976 The condensed tannins of pasture legume species. *Phytochemistry* 15 1407–1409.

Kaiser C J, Heath M E 1990 Big trefoil: A new legume for pastures on fragipan soils. In: *Advances in New Crops*. (Proc 1st Int Symp on New Crops: Research Development and Economics Timber Press, Portland, Oregon, USA), eds Janick J & Simon J E, Purdue Univ p 191.

Kelman W M, Tanner G J 1990 Foliar condensed tannin levels in lotus species growing on limed and unlimed soils in South-Eastern Australia. *Proc N Z Grassland Assoc* 52, 51–54.

Lees G L, Suttill N H, Gruber M Y 1993 Condensed tannins in sainfoin: a histological and cytological survey of plant tissues. *Can J Bot* 71 1147–1152.

Levin D A 1976 The chemical defenses of plants to pathogens and herbivores. *Ann Rev Ecol Syst* 7 121–159.

Lowther W L, Manley T R, Barry T N 1987 Condensed tannin concentrations in *Lotus corniculatus* and *L pedunculatus* cultivars grown under low soil fertility conditions. *NZ J Agric Res* 30 23–25.

Mantell S H, Smith H 1983 Cultural factors that influence secondary metabolite accumulations in plant cell and tissue cultures. In: *Plant Biotechnology*, eds Mantell S H & Smith H, Cambridge University Press, Cambridge, UK, pp 75–108.

Ross M D, Jones W T 1974 Host in cattle XL. Variation in flavanol content. In: *Lotus*. *NZJ Agric Res* 17 191–195.

Sarkar S H, Howarth R E 1976 Specificity of the vanillin test for flavanols. *J Agric Food Chem* 24 317–320.

Smith C W, Niles G A, Schuster M F 1990 registration of 11 upland cotton germplasm lines having elevated levels of condensed tannins. *Crop Sci* 30 1374–1375.

Terrill T H, Rowan A M, Douglas G B, Barry T N 1992 Determination of extractable and bound condensed tannin concentrations in forage plants, protein concentrate meals and cereal grains. *J Sci Food Agric* 58 321–329.

Watterson J J, Butler L G 1983 Occurrence of an unusual leucoanthocyanidin and absence of proanthocyanidins in sorghum leaves. *J Agric Food Chem* 31 41–45.

J. Sci. Fd Agric. 1972, **23**, 1151–1156

Lucerne Tannins

I. Content and Composition during Growth

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Composition and content of polyphenols of lucerne leaf and stalk were investigated during growth depending on the time of season at one growth stage.

(+)-Gallocatechin, (−)-epigallocatechin gallate, (−)-epigallocatechin, (−)-epicatechin, (−)-gallocatechin gallate, gallic acid, gallotannin and shikimic acid were estimated by qualitative chromatographic analyses on Whatman Chromedia paper SG-81. The content of some polyphenol groups was determined by quantitative analyses and amounts were found of 0.06 to 0.50%, gallic acid; 1.10 to 2.36%, gallotannins; 0.31 to 1.10%, catechins in the dry matter of lucerne depending upon growth stage and cuts. Polyphenoloxidase activity from 7.80 to 18.70 units was established in the investigated samples.

1. Introduction

The most frequently investigated polyphenols do not include substances containing nitrogen or triterpenoids, such as saponins and steroids, but they are the compounds that exist in nature and have the physiological influence due to their phenolic nature. This phenolic nature of the polyphenolic compounds is the factor conditioning toxicity. According to Cruickshank and Perrin,[1] Goodman, Kiraly and Zaitlin,[2] Herrmann[3] and Kuć[4] resistance of some plant varieties to certain diseases or pathogenic organisms depends on the content of natural tannins or their polyphenols in them.

According to Tamir and Alumot[5] tannins isolated from green carob beans had a significant inhibitory effect on the digestive enzymes trypsin, lipase and α-amylase.

Owing to their characteristics of coprecipitation with proteins, tannins diminish the biological value of the consumed food proteins in the intestinal tract. Protein and tannin interaction depends upon the conditions of forming hydrogen bonds between hydroxyl groups of polyphenols and tannins and the carbonyl groups of peptide bonds of proteins.[6] Covalent and ionic bonds can also be formed by the protein–polyphenol reaction but such a reaction is far less probable.

Because of the nature of tannins and the considerable amounts present in lucerne (about 3%), as well as the influence they have on animal nutrition, the aim of these investigations was to establish both the quantity and the composition of tannic substances in lucerne.

NO, 10, Oct.

2. Experimental

2.1. Material

Medicago sativa L. Panonia cultivar, the second cutting in three growth stages (20, 40 and 60 cm in height) and one growth stage (50 cm in height) by cuts within one growing season, was used as the experimental material.

Samples in duplicate were taken immediately after harvesting. The leaves were separated from the stalk and the weight ratio was determined on the basis of dry matter. The duplicate sample was prepared for analysis of the polyphenol substances.

2.2. Extraction

Homogeneous fresh plant material was weighed (approx. 15 g), macerated under nitrogen with a little water and extracted twice with distilled water (50 ml) at 95 °C under a reflux condenser for 40 min. The atmosphere above the extract was saturated with nitrogen from time to time. Combined extracts were cooled and centrifuged at 4.6×10^3 **g** to obtain clear supernatant. The extract was treated twice with 2-octane (50 ml) with vigorous stirring to remove the interfering substances, isohumulones. The 2-octane phase was isolated 5 min later and discarded. The water phase was transferred into a stirrer, mixed with 5 g of insoluble, crosslinked polyvinylpyrrolidone absorbent, Polyclar AT (Antara Chemical Division of General Aniline and Film. Corp., New York) with gentle stirring for 20 min. After standing for 10 min the liquid phase was discarded and the solid phase of the absorbent was extracted successively with three portions of 30 ml of hot ethyl acetate. Mixed ethyl acetate extracts were dried over anhydrous sodium sulphate and evaporated to a small volume at 45 °C in the rotary vacuum evaporator under a gentle flow of nitrogen. The residue was quantitatively transferred to a 10-ml volumetric flask and stored at −10 °C until analysis.

2.3. Identification

Identification of the individual polyphenols was carried out using the specific spot tests. Since all reducing compounds produce a blue colour with ferric chloride and potassium ferricyanide, vanillin–HCl mixture was used, being more specific and able to separate flavanols from gallic acid and the substances producing gallic acid and non-polyphenolic substances after hydrolysis. Irradiation with u.v. and the colour reactions with ethylenediamines and 0.05 N-NaOH were also used.

For chromatographic separation the following mixtures were used as the solvent systems.

(i) (1)2-Butanol saturated with H_2O and (2) 2% CH_3COOH for two-dimensional chromatography.
(ii) (1) 6% CH_3COOH and (2) 2-butanol–CH_3COOH–H_2O (14:1:5) for two-dimensional chromatography.
(iii) 1-Butanol–CH_3COOH–H_2O (40:12:28).
(iv) 2-Amyl alcohol–85% formic acid–H_2O (100:23:77).

Both chromatographic separation and identification were done on Whatman Chromedia paper SG-81 by the ascending technique.

2.4. Determination

Quantitative determination of individual polyphenol groups was conducted by the successive separation of gallotannins, flavanols (condensed tannins) and free gallic acid.

2.4.1. Gallotannins

An aliquot of 5 ml of ethyl acetate extract was transferred into a 20-ml conical tube containing 5 ml of benzene. The white amorphous precipitate of gallotannins formed was centrifuged off at 6.6×10^3 **g**, the supernatant separated and the precipitate dried under nitrogen with gentle warming. It was then dissolved in 5 ml of 25% ethanol, the content of polyphenols was determined using the colorimetric method with Folin–Denis reagent according to the treatment recommended by Burns[7] for forages.

2.4.2. Flavanols

The content of flavanols (condensed tannins) was determined in the supernatant following the precipitation of gallotannins using vanillin–HCl reagent after the method of Bate-Smith and Lerner[8] for leucoanthocyanins and catechins.

2.4.3. Free gallic acid

Quantitative determination was performed using the method described by Oshima and Nakabayashi.[9] 5 ml of ethyl acetate extract of the plant material was evaporated to dryness and dissolved in 5 ml of butyl acetate, followed with addition of 5 ml of benzene to separate gallotannins and the substances containing esterified gallic acid by precipitation. The precipitate was separated by centrifuging at 8.1×10^3 **g** and the supernatant was decanted into a conical tube. The latter was evaporated to dryness at 45 °C under nitrogen and dissolved in 15 ml of 25% ethanol. 10 ml of the mixture of 35% HCl and 30% formaldehyde (1:2) was added to the solution to produce precipitation of flavanols. One hour after the addition of the HCl–formaldehyde mixture the catechin precipitate was separated by centrifuging at 8.1×10^3 **g** and the content of gallic acid was determined in the supernatant by Folin–Denis reagent, after the method of Burns.[7]

2.4.4. Polyphenol oxidase

Polyphenol-oxidase activity was determined in all the leaf and stalk samples of lucerne using the method of Margna.[10]

3. Results and discussion

Two-dimensional paper chromatography, using the solvent systems (i), (ii), (iii) and (iv), of a 25% ethanolic solution of gallotannins gave one significant spot after staining with $FeCl_3$ and u.v. irradiation. Hydrolysis of the residue after being evaporated with 25% ethanolic solution in 0.7 N-HCl and examination gave the positive test for glucose.

Flavanols (condensed tannins) were identified from the ethyl acetate extracts by two-dimensional chromatography with the solvent systems (i) and (ii) and one-way chromatography using the solvent systems (iii) and (iv). The separated spots under

u.v. light showed dark blue fluorescence and after treatment with 0.05 N-NaCH were identified as (–)-epigallocatechin gallate, (–)-epigallocatechin, (+)-gallocatechin, (–)-epicatechin and (–)-gallocatechin gallate.

Free gallic acid, as well as catechin, was identified employing the solvent systems (ii) and (iii) for developing, and the best resolution from the accompanying catechins was obtained with the mixture of 2-amyl alcohol–85% formic acid–water (100:23:77).

Paper chromatographic studies using the solvent systems (i), (ii), (iii) and (iv) and the results of resolution as well as R_F values of the individual constituents are illustrated in Table 1.

TABLE 1. R_F values of individual polyphenols[a]

Constituents	R_F in system (i)		R_F in system (ii)		R_F in system (iii)	R_F in system (iv)
	(1)	(2)	(1)	(2)		
(+)-gallocatechin	0.50	0.30				0.40
(–)-epigallocatechin gallate	0.84	0.60			0.87	
(–)-epigallocatechin			0.68	0.70		
(–)-epicatechin	0.40	0.20	0.40	0.45	0.52	
(–)-gallocatechin gallate	0.45	0.87	0.50	0.64		0.28
Gallic acid				0.90	0.60	0.65
Gallotannin	0.10	0.20	0.15	0.70		
Shikimic acid[b]					0.35	

[a] R_F values of identified lucerne polyphenols given in Table 1 were compared with standard substances.

[b] Presence of shikimic acid was only found in some leaf samples and it was identified according to R_F values given in literature.

Certainly, gallotannins are the complex mixture determined by chromatography of some samples of ethyl acetate extracts. Such chromatographs gave a number of the spots identified but not characterised as gallotannins. The dissolved precipitate of gallotannin, when chromatographed afterwards, only gave one significant spot. Haworth[11] obtained similar results in chromatography of the precipitated gallotannins from Chinese tannin.

Condensed tannins were clearly identified according to their R_F values as compared to those obtained with the standard substances and literature data. A great number of detected catechins, all as flavan-3-ols, were probably transformed into non-hydrolysable tannins by enzyme activity or heating with water during extraction. It is possible only if the 6th- and/or 8th-positions are free. Both the ether and methanol of condensed tannins extract yielded only three flavanols, i.e. (–)-epicatechin-3-gallate, (–)-epicatechin and (+)-epicatechin gallate.

Free gallic acid was also detected on paper chromatographs of ethyl acetate extracts of lucerne leaf. Resolution of the combined spots of catechin and blue–violet spot of gallic acid identified under u.v. irradiation following chromatography with the solvent system 1-butanol–CH_3COOH–water was conducted by elution of the cut spots with ethyl acetate and rechromatography using the mixture of 2-amyl alcohol–formic acid–

water. The obtained R_F value of 0.60 was compared to the value of the pure gallic acid.

Quantitative values for polyphenols estimated in lucerne are illustrated in Tables 2 and 3.

TABLE 2. Content of polyphenol groups (% of dry matter) of lucerne depending on the stage of growth (height in cm)

Sample	Total tannic substances	Free gallic acid	Gallo-tannins	Flavanols	Polyphenol oxidase activity PphOx (mg ascorbic acid)
Leaf (cm)					
20	3.75	0.50	2.30	0.80	11.63
40	3.52	0.22	2.07	1.10	8.75
60	3.50	0.10	2.36	0.80	7.80
Stalk (cm)					
20	2.00	0.20	1.35	0.40	18.70
40	1.82	0.13	1.10	0.31	12.52
60	1.50	Trace	1.38	—	10.85
Whole plant (cm)					
20	3.05	0.38	1.92	0.64	14.46
40	2.76	0.18	1.74	0.77	11.22
60	2.47	0.06	1.85	0.40	9.32

TABLE 3. Content of individual polyphenol groups of lucerne (as % of dry matter) depending on the time of season at one growth stage (50 cm high)

Sample	Total tannic substances	Free gallic	Gallo-tannins	Flavanols	Polyphenol oxidase activity PphOx (mg ascorbic acid)
Leaf					
I–25.4. 1970	3.57	0.25	2.73	0.30	16.24
II-29.5. 1970	3.52	0.10	2.45	0.90	8.03
III–22.6. 1970	3.42	0.11	2.22	1.05	5.17
IV–17.7. 1970	3.19	0.08	2.03	1.12	5.03
Stalk					
I–25.4. 1970	2.09	0.14	1.44	0.40	18.45
II–29.5. 1970	1.93	0.09	1.47	0.41	11.57
III–22.6. 1970	1.87	Trace	1.39	0.45	10.38
IV–17.7. 1970	1.66	—	1.25	0.38	9.45
Whole plant					
I–25.4. 1970	2.97	0.18	2.20	0.34	16.80
II–29.5. 1970	2.75	0.09	2.03	0.75	9.38
III–22.6. 1970	2.75	0.05	1.75	0.70	7.44
IV–17.7. 1970	2.38	0.04	1.64	0.76	7.25

On the basis of the results obtained a markedly high content of gallic acid was reported, decreasing from 0.38 to 0.06% with plant maturing, which is surprising. The activity of oxidases as expected was lower in the later stages; therefore, it could be expected[12] that the content of free gallic acid increases with the growth of the plant. However, as the same procedure for determination was used for all the samples, i.e. precipitation of catechins in an acid medium with formaldehyde, followed by the determination of gallic acid present with Folin–Denis reagent, it could only be concluded that de-esterification, i.e. hydrolysis of the gallified catechins followed by liberation of gallic acid, occurred during formation of the precipitate which would account for the increase.

However, it is certain that non-esterified gallic acid exists in lucerne tissue and that its content decreases with the plant maturing.

The content of gallotannins and condensed tannins does not show considerable changes in the quantities during the growth of plant. Slight variations are not significant for gallotannins, whereas it can be considered that flavanols slightly decrease, particularly from the second to the last studied stage of lucerne growth.

The content of the total tannic substances, determined by Folin–Denis reagent from the ethylacetate extracts, without fractionation, decreases in both the leaf and the stalk, as well as in the whole plant during the year, by cuts, from 3.57 to 3.19% for the leaf, 2.09 to 1.66% for the stalk and from 2.97 to 2.38% for the whole plant.

Free gallic acid, gallotannins and total tannic substances have the same tendency of decreasing in lucerne during the year, by cuts, but the content of catechin increases from the first to the fourth cutting in the whole plant except in stalk in fourth cutting where the value is slightly decreased.

Polyphenol oxidase activity decreases both during the growth of the plant and by cuts. The polyphenol oxidase activity was determined on the suspension of the plant material, not the water extracts. Based on the fermentative oxidation of ascorbic acid in the presence of the specific substrate of polyphenol oxidase, pyrocatechin, the method has yielded good results of excellent reproducibility.

References

1. Cruickshank, I. A. M.; Perrin, D. R. *Biochemistry of Phenolic Compounds* (Harborne, J. E., ed.) Academic Press, New York. 1964, p. 511.
2. Goodman, R. N.; Kiraly, Z.; Zaitlin, H. *The Biochemistry and Physiology of Infectious Plant Disease* Van Nostrand, Princeton 1967, p. 187.
3. Herrmann, K. *Planta med.* 1962, **10**, 29.
4. Kuć, J. *A. Rev. Microbiol.* 1966, **20**, 337.
5. Tamir, M., Alumot, E. *J. Sci. Fd Agric.* 1969, **20**, 199.
6. Gustavson, K. H. *J. Polym. Sci.* 1954, **12**, 317.
7. Burns, R. E. *Rep. Ga. agric. exp. Stn.* 1963, p. 5.
8. Bate-Smith, R. C.; Lerner, N. H. *Biochem. J.* 1954, **58**, 126.
9. Oshima, Y., Nakabayashi, T. *J. Agric. Chem. Soc., Japan*, 1952, **26**, 377.
10. Margna, U. V. *Biohemija* 1964, **29**, 420.
11. Haworth, R. D. *The Chemistry of the Tannins.* (University of Sheffield, England), *Adv. Sci.* 1963, **19**, 396.
12. Zaprometov, M. N.; Buhlaeva, B. Yu. *Dokl. Akad. Nauk SSSR* 1963, **151** (1), 231.